# Targeted immunotherapy for ovarian cancer

Leffers, Ninke

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Stellingen behorende bij het proefschrift

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1. Ondanks hun immunosuppressieve karakter is de aanwezigheid van regulatoire T-cellen in de tumor niet per definitie voorspellend voor een ongunstige prognose in eierstokkanker.
   *Dit proefschrift*

2. De analyse van genexpressieprofielen is een waardevolle methode om te bepalen welke kenmerken van een tumor bijdragen aan de rekrutering van T-cellen.
   *Dit proefschrift*

3. P53-specifieke autoantistoffen zijn prognostisch irrelevante nevenproducten van p53-overexpressie.
   *Dit proefschrift*

4. Vaccinatie met het p53-SLP® vaccin leidt bij patiënten met eierstokkanker tot de activatie van p53-specifieke helper T-cellen.
   *Dit proefschrift*

5. Een adjuvant draagt in belangrijke mate bij aan zowel werking als bijwerking van een vaccin.
   *Dit proefschrift*

6. Er zijn vooralsnog geen immunologische parameters geïdentificeerd die onomstotelijk correleren met de klinische effectiviteit van een therapeutisch vaccin.
   *Dit proefschrift*

7. Met een korte follow-up worden lange termijn bijwerkingen gemist.
   *Dit proefschrift*

8. De relatie van MHC klasse I / $\beta_2$-microglobuline co-expressie met andere componenten uit het 'antigen processing and presentation pathway' suggereert een gecoördineerde regulatie van de expressie van coderende genen.
   *Dit proefschrift*

9. De sleutel tot succes van antigeen-specifieke immunotherapie ligt in een combinatie met medicatie die doelgericht immuunontsnappings-mechanismen van de tumor aanpakt.
   *Dit proefschrift*

10. Science never solves a problem without creating ten more.
    *George Bernard Shaw*

11. A scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die and a new generation grows up that is familiar with it.
    *Max Planck*

12. De uitspraak "great minds think alike" is slecht te verenigen met wetenschappelijke innovatie.

13. Medicine, the only profession that labours incessantly to destroy the reason for its existence.
    *James Bryce*

14. Om als arts ontwikkelingen in het veld op waarde te kunnen schatten, dient de competentie 'kritisch beschouwen van medisch-wetenschappelijk werk' tijdens de (vervolg)opleiding goed belicht te worden.

*Ninke Leffers*
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# Targeted Immunotherapy for Ovarian Cancer

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**Ninke Leffers**

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**Promotores:**

Prof. dr. H.W. Nijman
Prof. dr. C.A.H.H. Daemen
Prof. dr. A.G.J. van der Zee

**Beoordelingscommissie:**

Prof. dr. G.G. Kenter
Prof. dr. S.P. Schoenberger
Prof. dr. P.H.B. Willemse

**Paranimfen**: Dr. E. Dekker

[illegible] C. de Vrieze - Barends

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# Chapter 1

## General introduction

Partly based on:
Multimodality treatment warranted for ovarian cancer: immunotherapy, a prerequisite to improve prognosis for this vicious disease.

Leffers N, Daemen T, van der Zee AGJ, Nijman HW.

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## OVERVIEW OF CHAPTER

This chapter is a general introduction to the topics covered by this thesis. The main aim of this thesis was to investigate the achievability of immunotherapy for ovarian cancer.

The first section in this chapter is an introduction on ovarian cancer, which will clarify the necessity of exploring new therapeutic options for ovarian cancer. Next, the basic principles of tumour immunology are outlined. Thirdly, strategies to influence the immune system to reject and destroy tumours, i.e. immunotherapy, are discussed with an emphasis on therapeutic options especially relevant to ovarian cancer. Lastly, a brief overview of the contents of this thesis is given.

### Ovarian Cancer

Ovarian cancer refers to malignant disease involving the ovaries. The majority of malignant ovarian tumours are epithelial carcinomas (80-90%). However, tumours may also arise from the stromal compartment of the ovary and from germ-cells present within the ovary. In addition, a small minority of ovarian malignancies are metastases from other primary tumours. The projects in this thesis deal with epithelial ovarian cancer, and any statements made about ovarian cancer should be regarded as specific for epithelial ovarian cancer only.

In the year 2006, ovarian cancer was the eighth most common invasive tumour in Dutch females (1092 new cases according to the Dutch Cancer Registry), with a life time risk of 1:80. Although not the most frequently diagnosed type, ovarian cancer is the most common cause of death from gynaecological malignancies (2006: 1009 deaths). This high mortality is best attributed to the lack of specific symptoms, which in the majority of patients results in diagnosis in an advanced stage of disease.

Ovarian cancer is generally treated with cytoreductive surgery followed by chemotherapy. As the amount of residual tumour tissue after surgery is an important prognostic factor in ovarian cancer, an attempt is made to completely remove all malignant tissue. Subsequently, patients are treated with adjuvant chemotherapy. Platinum-based agents, have been used in the treatment of ovarian cancer since the late seventies. In the mid-nineties, taxanes, e.g. paclitaxel or docetaxel, were added to the regimen as the combination of taxanes and platinum-based agents result in increased progression-free and disease-specific survival in patients with (in)completely debulked advanced stage ovarian cancer (1). Despite these changes in chemotherapy, five-year disease-specific survival of ovarian cancer patients has only modestly improved over the last decades and remains low at ~40%. In an attempt to improve prognosis, new treatment modalities are being explored. One of these strategies under exploration is immunotherapy, a strategy which aims at the induction of tumour-specific immune responses.

**Tumour Immunology**

As early as in the beginning of the 20th century, it was postulated that the immune system may play an important role in the prevention and treatment of cancer. However, not until some fifty years later, this notion was further elaborated upon by Macfarlane Burnet. He postulated that a mechanism, most probably of immunological origin, exists that inactivates or eliminates potentially malignant cells. This phenomonen was named the cancer immunosurveillance hypothesis (2-4).

Malignant cells are the result of genetic changes that may arise during cell divisions. These genetic changes may result in the expression of so-called tumour antigens, which make malignant cells immunologically distinguishable from normal cells. As for most human tumours with unknown aetiology, no tumour-specific antigens have been identified for ovarian cancer. Nevertheless, antigenic targets for the immune system do exist in the form of tumour-associated antigens (i.e., self-antigens with an altered expression in tumour cells as compared with normal body cells) (5). Several types of tumour-associated antigens have been identified in ovarian cancer, for example, up-regulation and/or mutation in oncogenes and/or tumour-suppressor genes (Her-2/Neu and p53), expression of cancer testis antigens (NY-ESO-1, MAGE and LAGE-1) and glycolipid antigens (MUC1 and CA125) (6). None of the antigens identified thus far are universally expressed by epithelial ovarian malignancies. Moreover, even in a single individual patient heterogeneous antigen expression within a single tumour nodule or between primary tumours and metastases may exist.

Tumour antigens may be recognised by cells of the adaptive immune system, i.e. B-lymphocytes and T-lymphocytes. An increasing body of evidence indicates that the presence of tumour-infiltrating lymphocytes is associated with more favourable prognosis of ovarian cancer (7-9). The presence of intra-tumoral lymphocytes and the associated favourable prognosis are generally ascribed to tumour-specific immune responses and form the rationale for the development of immunotherapy for ovarian cancer. Despite the fact that anti-tumour immune responses have been described for ovarian cancer patients (10-15), these are apparently often not sufficient to fully eradicate tumours. Since the first postulation of the cancer immunosurveillance hypothesis, it has become clear that the intricate interaction between immune system and tumour cells not only protects against the occurrence of malignancies (immunosurveillance – elimination phase), but also promotes the selection of tumour cell variants with characteristics that facilitate escape from immune attack (equilibrium phase). Ultimately this results in the complete escape of tumour cells from the immune system, resulting in uncontrolled tumour growth (escape phase). This three step interaction process between tumour cells and the immune system is referred to as cancer immunoediting (16).

## Immunotherapy

With immunotherapy, an attempt is made to stimulate the immune system to reject and destroy tumours by administration of an immunologically active compound. Similar to immunisation in the preventative setting, passive and active immunisation strategies exist. In this thesis, active targeted immunotherapy is discussed and often simply referred to as immunotherapy.

Ideally, an immunotherapeutic compound is highly immunogenic, easily manufactured and universally applicable. Antigen-specific immunotherapy strategies include administration of: proteins/peptides with or without adjuvant; dendritic cells (DCs; transfected with DNA- or RNA-encoding tumour antigens, or loaded with peptides, whole proteins or tumour lysates); recombinant viruses encoding tumour antigens; and autologous or allogeneic tumour cells (6). Antigen-restricted strategies have the distinct advantage that antigen-specific immune responses can be easily monitored as a tool to improve the vaccine or immunization strategy. Yet, short-peptide vaccines induce either T helper or cytotoxic T-cells and are limited to patients with certain HLA genotypes corresponding to the peptide used. By contrast, whole-protein or long-peptide vaccines may contain epitopes for both T helper and cytotoxic T-cells and can be applied irrespective of HLA genotype. DC vaccines are laborious in production and restricted to individual patients, but have the advantage that DCs are highly efficient potent immunostimulatory cells. Type and maturation status of DCs are issues to be solved in this vaccination approach. An important challenge facing viral vector vaccines is antigenic competition between the target antigen and the viral vector itself. Tumour cell-derived vaccines and vaccines consisting of DCs loaded with tumour lysates contain multiple unknown antigens that, on the one hand may include patient-specific strong antigenic epitopes, but on the other hand may contain epitopes for which tolerance was created, thus diluting an effective immune response. Which vaccine type will ultimately result in the best trade-off between clinical applicability and immunogenicity and/or clinical efficacy remains to be elucidated.

## OUTLINE OF THIS THESIS

**Chapter two** discusses the prognostic significance of three different types of T-lymphocytes in ovarian cancer. The presence of intra-tumoural cytotoxic, memory and regulatory T-lymphocytes was determined in 306 ovarian cancer patients. Cytotoxic and memory T-lymphocytes may act as anti-tumour effector cells, whereas regulatory T-lymphocytes suppress proliferation and anti-tumour activity of effector lymphocytes. Next to primary ovarian tumour tissue, the presence and prognostic significance of these tumour-infiltrating lymphocytes was determined in omental metastases.

**Chapter three** describes the prognostic significance of p53-overexpression, a tumour antigen, in 329 ovarian cancer patients. In addition, humoural anti-p53 immunity was evaluated in sera of 233 patients. Lastly, the prognostic influence of p53-overexpression was evaluated combined with MHC class I down-regulation, an immune escape mechanism frequently utilized by tumour cells.

**Chapter four** further portrays down-regulation of components of the MHC class I dependent antigen processing and presentation pathway as immune escape mechanism in ovarian cancer. Down-regulation of components of this pathway may result in impaired antigen presentation to T-lymphocytes, thus providing tumour cells with a means of escaping recognition and destruction by cells of the adaptive immune system.

In **chapter five** an attempt was made to elucidate what factors determine whether tumour-infiltrating lymphocytes are present, comparing gene profiles and pathway activation between patients with high and low numbers of intra-tumoural T-lymphocytes. Differences were subsequently validated in a larger population, previously analyzed in chapter two for tumour-infiltrating lymphocytes.

In **chapter six**, a systematic overview is given of antigen-specific active immunotherapy strategies in ovarian cancer. Furthermore, recommendations are made to improve quality of future immunotherapy studies in ovarian cancer.

The existence of a p53-specific T-cell repertoire in ovarian cancer patients (11) combined with the results of chapter three substantiate p53 as an interesting target for immunotherapy of ovarian cancer. **Chapter seven** describes the results of a phase II study evaluating the immunogenicity of a vaccine targeting p53 in recurrent ovarian cancer patients. Twenty patients were immunized with p53 synthetic long peptides (p53-SLP®) in Montanide ISA-51. P53-specific immune responses as well as clinical responses were evaluated.

In **chapter 8** long-term immunological and clinical follow-up of patients participating in the above-mentioned immunotherapy trial is described. Long-term clinical benefits have been described after vaccination, therefore it was evaluated whether this also applies to ovarian cancer patients treated with the p53-SLP vaccine. Furthermore, it was investigated whether vaccine-induced p53-specific T-lymphocytes survive chemotherapy secondary to immunotherapy.

Lastly, **chapter 9** summarizes the research described in previous chapters and provides suggestions for further research of immunotherapy in ovarian cancer.

## REFERENCES


(1) McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996;334:1-6.

(2) Burnet FM. Immunological factors in the process of carcinogenesis. Br Med Bull 1964;20:154-158.

(3) Burnet FM. The concept of immunological surveillance. Prog Exp Tumor Res 1970;13:1-27.

(4) Burnet M. Cancer: a biological approach. I The processes of control. Br Med J 1957;1:779-786.

(5) Finn OJ. Cancer immunology. N Engl J Med 2008;358:2704-2715.

(6) Sabbatini P, Odunsi K. Immunologic Approaches to Ovarian Cancer Treatment. J Clin Oncol 2007;25:2884-2893.

(7) Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942-949.

(8) Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005;102:18538-18543.

9) Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348:203-213.

(10) Chiriva-Internati M, Wang Z, Salati E, Timmins P, Lim SH. Tumor vaccine for ovarian carcinoma targeting sperm protein 17. Cancer 2002;94:2447-2453.

(11) Lambeck A, Leffers N, Hoogeboom BN, et al. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007;121:606-614.

(12) Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 1995;181:2109-2117.

(13) Ioannides CG, Fisk B, Jerome KR, Irimura T, Wharton JT, Finn OJ. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol 1993;151:3693-3703.

(14) Qian F, Gnjatic S, Jager E, et al. Th1/Th2 CD4+ T cell responses against NY-ESO-1 in HLA-DPB1*0401/0402 patients with epithelial ovarian cancer. Cancer Immun 2004;4:12.

(15) Yamada A, Kawano K, Harashima N, et al. Study of HLA class I restriction and the directed antigens of cytotoxic T lymphocytes at the tumor sites of ovarian cancer. Cancer Immunol Immunother 1999;48:147-152.

(16) Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol 2002;3:991-998.

# Chapter 2



## Prognostic significance of tumour-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer

Leffers N*, Gooden MJM*, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, Boezen HM, van der Zee AGJ, Daemen T, Nijman HW

** Both authors contributed equally*

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## ABSTRACT

### Purpose

Ovarian cancer patients with intra-tumoural $CD3^+$ T-lymphocytes in primary tumour tissue have a better prognosis. This study aims to analyze the presence and relative influence of three important T-lymphocyte subsets, tumour-infiltrating $CD8^+$ cytotoxic T lymphocytes (CTL), $CD45R0^+$ memory T-lymphocytes and $FoxP3^+$ regulatory T-lymphocytes (Treg), in primary tumour tissue and omental metastases of patients with ovarian cancer.

### Experimental Design

The number of $CD8^+$, $CD45R0^+$ and $FoxP3^+$ T-lymphocytes was determined by immunohistochemistry on a tissue micro array containing ovarian tumour tissue and/or omental metastases obtained at primary debulking surgery from 306 FIGO stage I-IV ovarian cancer patients. Immunohistochemistry data were correlated to clinicopathological parameters and survival data.

### Results

High number of $CD8^+$ CTL and a high $CD8^+/FoxP3^+$ ratio in ovarian-derived tumour tissue were associated with increased disease specific survival and proved to be independent prognostic factors in multivariate analyses. In advanced stage patients, the presence of $CD8^+$ CTL, $CD45R0^+$ memory T-lymphocytes, $FoxP3^+$ Treg or a high $CD8^+/FoxP3^+$ ratio in ovarian-derived tumour tissue was associated with an increased disease-specific survival in univariate analysis, as was the presence of $CD45R0^+$ memory T-lymphocytes and $FoxP3^+$ Treg in omental metastases. Furthermore, in advanced stage patients $CD8^+$ cytotoxic and $FoxP3^+$ regulatory T-lymphocytes infiltrating ovarian-derived tumour tissue were independent predictors of increased prognosis.

### Conclusions

T-lymphocytes infiltrating primary and metastatic ovarian cancer sites are associated with improved prognosis. These associations are especially distinct in advanced stage patients, underlining the potential for immunotherapy as a broadly applicable therapeutic strategy.

## INTRODUCTION

Ovarian cancer is the most frequent cause of death from gynaecological malignancies, with an overall mortality of 60%. Due to its lack of specific symptoms, 70% of patients present with advanced disease. Initial treatment consists of cytoreductive surgery and platinum based chemotherapy. Even when initial treatment is successful, many patients relapse. There is no curative secondary treatment in case of residual disease after chemotherapy or relapse (1;2). Therefore, new therapeutic strategies are under investigation (3). Immunotherapy is one of these strategies and has yielded especially promising results in fundamental and animal research. However, clinical applications have only shown limited efficacy. Further knowledge is necessary to develop strategies to increase clinical efficacy of immunotherapy.

Tumour-infiltrating lymphocytes (TIL) were found to correlate with improved prognosis in several types of cancer, among which ovarian cancer (4-9). The presence of TIL is considered a reflection of the immune response to the tumour. However, not all T-lymphocyte subsets contribute equally to this observed positive effect on prognosis. Invasion by cytotoxic T lymphocytes (CTL) has been found to be advantageous in ovarian cancer (7) and several other types of cancer (10-13). The role of $CD4^+$ T lymphocytes is ambiguous. Non-regulatory $CD4^+$ helper T lymphocytes, especially of the Th1 subset, are considered to be beneficial in cancer in general (14). Regulatory $CD4^+$ T-lymphocytes (Treg) have a physiological function in preventing autoimmunity. They may induce peripheral tolerance, and, in doing so, suppress immune responses. In cancer, Treg traffic to tumours as a result of chemokines produced by tumour cells and micro-environmental macrophages (15). There, they suppress effector T-lymphocytes by secreting transforming growth factor β (TGF-β) and interleukin 10 (IL-10) or by direct cell-cell contact (15). Treg have been found to unfavourably influence prognosis in ovarian cancer (15;16). From a functional point of view, the ratio between effector T-lymphocytes and regulatory T-lymphocytes may be even more interesting, as was demonstrated by Sato et al. (7) who showed that high ratios of CTL/helper T-lymphocytes and CTL/Treg were of prognostic significance, whereas no significant association between the number of tumour infiltrating T-lymphocytes in general or subsets (Treg / helper T-lymphocytes) and overall survival were found in ovarian cancer. A less well studied T-lymphocyte subset are memory T-lymphocytes, which may arise after the initial immune response against an antigen. In cancer, memory T-lymphocytes mediate long-term immunity against tumours (17). The presence of intra-tumoural memory T-lymphocytes is associated with increased survival in hepatocellular (18) and colorectal cancer (5;17). To our knowledge, no studies have been published investigating the presence of memory T-lymphocytes in ovarian cancer. In summary, patients with tumour-infiltrating T-lymphocytes mount an immune response against their tumour, the

success of which seems to depend on the relative concentrations of different T-cell subtypes.

No reports have been published describing possible differences in T-lymphocyte infiltration patterns between primary tumour sites and metastases. The omentum is a frequent site for metastases in ovarian cancer, due to the displacement of tumour cells by the peritoneal fluid stream. We decided to study the presence of infiltrating T-lymphocytes in omental metastases, which was greatly facilitated by the fact that omentectomy is a routine part of cytoreductive surgery. We compared the results with infiltrating T-lymphocytes in tissue, taken from the primary ovarian tumour.

Aims of the present study were to determine the presence of $CD8^+$ CTL, $CD45R0^+$ memory T-lymphocytes and $FoxP3^+$ Treg in primary tumour specimens and simultaneous omental metastases of patients with ovarian cancer and to determine their prognostic impact.

## METHODS

### Patients

As of 1985 the Department of Gynaecological Oncology at the University Medical Center Groningen (UMCG) keeps a computerized database of patients with malignant epithelial ovarian cancer treated at this hospital at any time point during the course of their disease, prospectively collecting information on clinicopathological characteristics and follow-up.

For this study, ovarian cancer patients were selected if primary surgery was performed by a gynaecological oncologist from the UMCG between May 1985 and June 2006 and if paraffin-embedded ovarian and omental tumour tissue was available.

Patients were staged according to FIGO classification (19). Tumours were graded and classified according to WHO criteria by a gynaecological pathologist (20). Adjuvant chemotherapy consisted of different platinum-based treatment regimens. Response to chemotherapy was evaluated according to WHO criteria (21). After treatment, patients were followed-up for at least 10 years with gradually increasing intervals. Follow-up data were completed for all patients until January 2007.

### Institutional Review Board Approval

For the present study, all relevant data were retrieved from our computerized database into a separate anonymous database. In this separate, password-protected database, patient identity was protected by study-specific, unique patient codes, which were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathological and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. According to Dutch law no approval from our IRB was needed.

### Tissue Micro arrays

Tissue micro arrays (TMA) were constructed as described in previous studies (22;23). In brief, paraffin-embedded tissue blocks containing tumour in ovarian and omental tissue, and corresponding haematoxylin & eosin (H&E) stained slides were retrieved from the pathology archives. Representative areas of tumour were marked on the H&E stained slides. Next, using these H&E slides for reference, four 0.6mm core biopsies were taken from each tumour specimen and arrayed on a recipient paraffin block using a tissue microarrayer (Beecher instruments, Silver Spring, Maryland, USA). Adhesion of cores to the recipient block was accomplished by placing the blocks in a 37°C oven for 15 min. For staining, 4µm sections were cut from each TMA block. H&E staining was performed to verify the presence of tumour in the arrayed samples.

**Immunohistochemistry**

TMA sections were stained with mouse monoclonal antibodies recognizing CTL (anti-CD8; Dako cytomation, Glostrup, Denmark), memory T-lymphocytes (anti-CD45R0 clone OPD4; Labvision, Fremont CA, USA) and Treg (anti-FoxP3 m22509, Abcam, Cambridge, UK). In brief, TMA sections were dewaxed in xylene and rehydrated using graded concentrations of ethanol to distilled water. After antigen retrieval, endogenous peroxidase activity was blocked by submersion of sections in a 0.3% $H_2O_2$ solution for 30 min. Sections were incubated with the primary antibody for 60 min at room temperature (dilutions: anti-CD8 1:20; anti-CD45R0 1:50; anti-FoxP3 1:100). Sections incubated with anti-CD45R0 and anti-FoxP3 were subsequently incubated with DAKO Envision+ for 30 min. For sections incubated with anti-CD8, $RAM^{po}$ (dilution 1:100) and $GAR^{po}$ (dilution 1:100) were used as secondary and tertiary antibodies respectively. The antigen-antibody reactions were visualized with 3,3'-diaminobenzidine or NovaRED™ (Vector Laboratories, Burlingame) for anti-FoxP3 staining. Sections were counterstained with haematoxylin.

**Scoring**

All stainings were scored independently by two observers. Observers had no prior knowledge of clinicopathological information. For the anti-CD8 and anti-CD45R0 staining, the number of cells with membrane staining within tumour epithelium was counted for each core. For the anti-FoxP3 staining, the number of cells with nuclear staining within tumour-islets in every core was counted. To obtain a high concordance rate with whole tissue slides, we decided that minimally two cores with a minimum of 20% tumour tissue had to be present on the TMA for a sample to be entered into analysis (23). Subsequently, we calculated the number of intra-tumoural cells per 0,283 $mm^2$ of tumour (i.e. one whole core consisting of 100% tumour tissue), to correct for differences in the amount of tumour tissue and to standardize the analysis. This calculation entailed dividing the total number of intra-tumoural cells in cores containing ≥20% of tumour by the total percentage of tumour tissue present in these cores.

**Statistics**

The distribution of all TIL subtypes was positively skewed. For further analysis, we therefore decided to categorize patients using the bottom tertile (p33) as a cut-off value (7). However, when the bottom tertile equalled zero, a subdivision based on the presence or absence of these cells was made for further analysis. Associations between clinicopathological characteristics and intra-tumoural T-lymphocytes were tested using the $\chi^2$ test. Differences in numbers of tumour-infiltrating T-lymphocytes between primary ovarian tumour tissue and omental metastases were tested using Wilcoxon Signed Ranks test. Disease-specific survival (DSS) was defined as date of surgery until death due to ovarian cancer or date of last follow-up. DSS was estimated using Kaplan Meier and Log Rank test was used to assess for survival differences between groups. Cox proportional hazards models were used for

multivariate analyses and were stratified for type of chemotherapy. Only variables that were significantly associated with DSS in the univariate analyses were entered into multivariate analyses. Survival analyses were performed in the total population as well as in a subgroup which included all advanced stage patients. For all tests, p-values <0.05 were considered significant. All p-values were tested two-sided. All statistical analyses were performed using SPSS 14.0 software package for windows (SPSS Inc., Chicago, IL, USA).



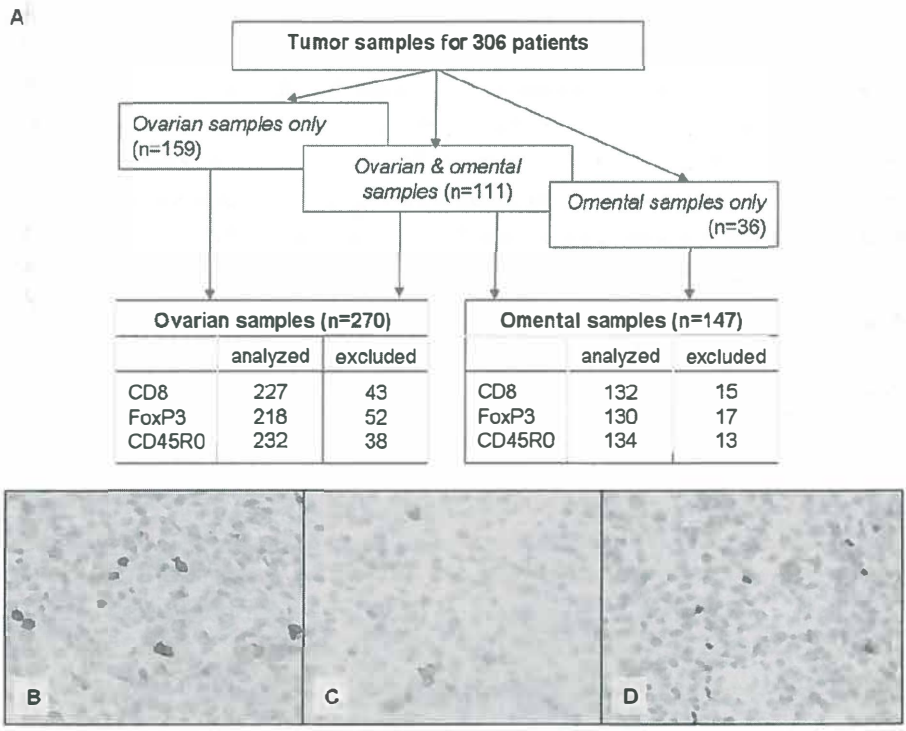


**Fig. 1** Immunhistochemical staining of tumor-infiltrating T-lymphocytes in ovarian cancer tissue. **A** Flow diagram showing the type of tissue analyzed and the percentage of patients lost from analysis. Patients were excluded from analysis when less than two tissue cores with at least 20% tumor were present for evaluation. **B** Intra-tumoral $CD8^+$ T-lymphocytes, **C** $CD45RO^+$ T-lymphocytes, **D** $FoxP3^+$T-lymphocytes at 400x magnification.

## RESULTS

### Study population

From a total of 306 ovarian cancer patients, sufficient paraffin-embedded ovarian and/or omental tissue containing epithelial ovarian carcinoma was available for construction of the TMA (figure 1a). Tumour-containing ovarian tissue was available from 270 patients. From 111 (41.1%) of these patients tumour-containing omental tissue was also available. Only tumour-containing omental tissue was available from an additional 36 patients. Clinicopathological characteristics of patients are summarized in Table 1. Forty-two patients did not receive chemotherapy, 28 of whom were diagnosed with FIGO stage I disease. The remaining 14 patients were either not fit or unwilling to receive chemotherapy. Of the patients treated with chemotherapy 89.5% received a platinum-based regimen. The median survival for all patients was 37.4 months with an estimated 5-year disease specific survival of 41%.

### Tumour-infiltrating T-lymphocytes

Intra-tumoural $CD8^+$, $CD45R0^+$ and $FoxP3^+$ T-cells were present in 91.2%, 47.0% and 53.2% of primary ovarian tissue, respectively (representative examples in figure 1b-d), while intra-tumoural $CD8^+$, $CD45R0^+$ and $FoxP3^+$ T-cells were present in 96.2%, 49.3% and 73.1% of omental metastases, respectively.

In both ovarian and omental derived tumour tissue, the number of tumour-infiltrating T-lymphocytes was positively associated with each other for all subtypes (Tables 2 and 3). Although the median number of tumour-infiltrating T-lymphocytes was higher for omental than ovarian tissue, no differences in intra-tumoural T-lymphocytes were observed between ovarian and omental tumour tissue within patients for whom both samples were available (data not shown).

### Association of tumour-infiltrating lymphocytes with clinicopathological parameters

Table 2 and 3 show the relationship of clinicopathological parameters to the presence of intra-tumoural TIL in ovary and omentum derived tumour tissue respectively. In ovarian tissue, the presence of $FoxP3^+$ cells was positively associated with advanced stage disease ($p=0.031$). Furthermore, patients with poorly differentiated tumours were more likely to have intra-tumoural $FoxP3^+$ cells compared to patients with well-differentiated tumours ($p=0.011$). Also, patients younger than 59 years at time of diagnosis were more likely to have a low $CD8^+/CD45R0^+$ ratio than older patients (data not shown: 48.5% vs. 34.3%, $p=0.036$).

In omental metastases, high $CD8^+$ T-cell numbers were associated with <2 cm residual disease after primary debulking surgery ($p=0.035$). A similar association was found for $FoxP3^+$ cells, which were more often present in patients with residual disease of <2cm ($p=0.028$).

**Survival analysis**

In agreement with expectations, well-known prognostic factors such as age ≥ 59 years, advanced stage disease, poorly differentiated tumours, serous tumours and ≥2 cm residual disease after primary debulking surgery were associated with a shorter disease specific survival (data not shown). Furthermore, univariate analysis of disease specific survival showed an initial survival advantage for patients with high

**Table 1** Clinicopathological characteristics and survival data

| | Total (n=306) | | Primary ovarian cancer (n=270) | | Metastatic omental – ovarian cancer (n=147) | |
|---|---|---|---|---|---|---|
| *Age (years)* | | | | | | |
| Mean (SD) | 57.2 | (13.5) | 56.8 | (13.8) | 59.6 | (12.2) |
| *DSS (months)* | | | | | | |
| Median (95% C.I.) | 37.4 | (25.6-49.2) | 45.4 | (31.1-59.8) | 20.4 | (14.3-27.3) |
| *FIGO Stage* | | | | | | |
| Stage I | 67 | (21.9%) | 67 | (24.8%) | | |
| Stage II | 24 | (7.8%) | 25 | (9.3%) | | |
| Stage III | 171 | (55.9%) | 145 | (53.7%) | 116 | (78.9%) |
| Stage IV | 42 | (13.7%) | 32 | (11.9%) | 30 | (20.4%) |
| Missing | 2 | (0.7%) | 1 | (0.4%) | 1 | (0.7%) |
| *Tumour type* | | | | | | |
| Serous | 171 | (55.9%) | 147 | (54.4%) | 105 | (71.4%) |
| Mucinous | 36 | (11.8%) | 35 | (13.0%) | 6 | (4.1%) |
| Endometroid | 41 | (13.4%) | 38 | (14.1%) | 13 | (8.8%) |
| Clear Cell | 21 | (6.9%) | 18 | (6.7%) | 7 | (4.8%) |
| Adenocarcinoma | 13 | (4.2%) | 10 | (3.7%) | 5 | (3.4%) |
| Mixed Tumours | 15 | (4.9%) | 14 | (5.2%) | 5 | (3.4%) |
| Other | 9 | (2.9%) | 8 | (3.0%) | 6 | (4.1%) |
| *Tumour Grade* | | | | | | |
| Grade I | 52 | (17.0%) | 51 | (18.9%) | 6 | (4.1%) |
| Grade II | 80 | (26.1%) | 76 | (28.1%) | 28 | (19.0%) |
| Grade III | 135 | (44.1%) | 113 | (41.9%) | 91 | (61.9%) |
| Undifferentiated | 14 | (4.6%) | 11 | (4.1%) | 9 | (6.1%) |
| Missing | 25 | (8.2%) | 19 | (7.0%) | 13 | (8.8%) |
| *Residual disease* | | | | | | |
| < 2 cm | 162 | (52.9%) | 157 | (58.1%) | 47 | (32.0%) |
| >= 2 cm | 123 | (40.2%) | 92 | (34.1%) | 94 | (63.9%) |
| Missing | 21 | (6.9%) | 21 | (7.8%) | 6 | (4.1%) |
| *Chemotherapy* | | | | | | |
| No chemotherapy | 42 | (13.7%) | 40 | (14.8%) | 12 | (8.2%) |
| Platinum-containing | 117 | (38.2%) | 104 | (38.5%) | 48 | (32.7%) |
| Platinum & taxane containing | 113 | (36.9%) | 96 | (35.6%) | 69 | (46.9%) |
| Other regimen | 27 | (8.8%) | 24 | (8.9%) | 16 | (10.9%) |
| Unknown | 7 | (2.3%) | 6 | (2.2%) | 2 | (1.4%) |

DSS = disease-specific survival; FIGO = International Federation of Gynaecology and Obstetrics.

numbers of intra-tumoural $CD8^+$ cytotoxic T-lymphocytes in ovarian-derived tumour tissue, which subsides after 10 years of follow-up (p=0.042, figure 2a). Disease-specific survival was not influenced by the presence of $CD45R0^+$ memory T-lymphocytes, $FoxP3^+$ regulatory T-lymphocytes in ovarian tissue. However, median DSS of patients with a high $CD8^+/FoxP3^+$ ratio in ovarian-derived tumour tissue was twice as high as for patients with a low $CD8^+/FoxP3^+$ ratio (50.0 vs. 23.0 months, p=0.014, figure 2b). For the $CD8^+/CD45R0^+$ ratio a trend was observed towards a longer median DSS in patients with a high ratio (51.7 vs. 30.9 months, p=0.056).

We subsequently repeated univariate survival analyses using a subgroup consisting of patients with advanced stage disease. Interestingly, in this subgroup a prolonged median DSS was observed for all T-lymphocyte subsets studied in ovarian-derived tumour tissue, except for the $CD8^+/CD45R0^+$ ratio (30.5 vs. 15.6 months, p=0.076), i.e. $CD45R0^+$ lymphocytes (n=155, 37.3 vs. 16.4 months, p=0.009, figure 2c), $CD8^+$ lymphocytes (n=150, 30.3 vs. 12.2 months, p=0.012, figure 2d), $FoxP3^+$ lymphocytes (n=149, 30.3 vs. 14.0 months, p=0.008, figure 2e) and a high $CD8^+/FoxP3^+$ ratio (n=146, 30.3 vs. 14.6 months; p=0.016, figure 2f). Similarly, univariate analysis of disease-specific survival differences based on the presence of TIL in omental metastases, which are by definition present only in patients with advanced stage disease, showed an increased survival in patients with intra-tumoural $FoxP3^+$ Treg (25.6 vs. 14.7 months, p<0.008, figure 2g) and $CD45R0^+$ memory T-lymphocytes (24.3 vs. 15.6 months, p=0.031, figure 2h).

Multivariate analysis was performed stratified for type of chemotherapy. For all patients as well as the advanced stage subgroup, the above-mentioned well-known prognostic factors and tumour-infiltrating T-lymphocytes were entered into the Cox proportional hazards model (table 4). In the analysis using all patients, $CD8^+$ lymphocytes and $CD8^+/FoxP3^+$ ratio were independent prognostic factors for DSS (HR 0.36, 95% C.I. 0.22-0.59, p<0.001; resp. HR 0.53, 95%C.I. 0.32-0.85, p=0.009), next to commonly acknowledged risk factors as stage of disease, differentiation grade and amount of residual tumour after primary debulking surgery. When analyzing only patients with advanced stage disease, a high number of $CD8^+$ cytotoxic T-lymphocytes in ovarian-derived tumour tissue was demonstrated to be an independent prognostic factor for longer DSS (HR 0.35, 95% C.I. 0.21-0.60, p<0.001), as was the presence of $FoxP3^+$ Treg (H.R. 0.55, 95%C.I. 0.34-0.88, p=0.013). Additionally, multivariate analysis was performed using the absolute number of tumour-infiltrating T-lymphocytes instead of the dichotomized variables (data not shown). Unlike $CD8^+$ lymphocytes, $FoxP3^+$ lymphocytes infiltrating tumour epithelium remained an independent factor for longer DSS (HR 0.937, 95%C.I. 0.88-1.00, p = 0.035).

**Table 2** Relationship of tumour-infiltrating lymphocytes in ovarian tissue to clinicopathological parameters and each other (N, %).

| | CD8⁺ T-lymphocyte | | | CD45R0⁺ T-lymphocyte | | | FoxP3⁺ T-lymphocyte | | |
|---|---|---|---|---|---|---|---|---|---|
| | lowest tertile | all others | p-value[a] | absent | present | p-value[a] | absent | present | p-value[a] |
| *Age (years)* | | | | | | | | | |
| <59 | 43 (38.7%) | 68 (61.3%) | 0.101 | 61 (55.0%) | 50 (45.0%) | 0.571 | 48 (44.9%) | 59 (55.1%) | 0.575 |
| ≥59 | 33 (28.4%) | 83 (71.6%) | | 62 (51.2%) | 59 (48.8%) | | 54 (48.6%) | 57 (51.4%) | |
| *FIGO stage* | | | | | | | | | |
| Stage I/II | 25 (33.3%) | 50 (66.7%) | 0.947 | 45 (59.2%) | 31 (40.8%) | 0.173 | 39 (57.4%) | 29 (42.6%) | **0.031** |
| Stage III/IV | 51 (33.8%) | 100 (66.2%) | | 77 (49.7%) | 78 (50.3%) | | 62 (41.6%) | 87 (58.4%) | |
| *Tumour type* | | | | | | | | | |
| Serous | 37 (30.3%) | 85 (69.7%) | 0.278 | 68 (52.7%) | 61 (47.3%) | 0.917 | 51 (42.1%) | 70 (57.9%) | 0.125 |
| Non-serous | 39 (37.1%) | 66 (62.9%) | | 55 (53.4%) | 48 (46.6%) | | 51 (52.6%) | 46 (47.4%) | |
| *Differentiation grade* | | | | | | | | | |
| Grade I/II | 39 (37.5%) | 65 (62.5%) | 0.208 | 56 (54.9%) | 46 (45.1%) | 0.513 | 55 (57.3%) | 41 (42.7%) | **0.011** |
| Grade III & undiff. | 32 (29.4%) | 77 (70.6%) | | 57 (50.4%) | 56 (49.6%) | | 43 (39.4%) | 66 (60.6%) | |
| *Residual disease* | | | | | | | | | |
| <2cm | 45 (36.0%) | 80 (64.0%) | 0.692 | 64 (48.9%) | 67 (51.1%) | 0.109 | 58 (50.4%) | 57 (49.6%) | 0.577 |
| ≥2cm | 28 (33.3%) | 56 (66.7%) | | 51 (60.0%) | 34 (40.0%) | | 39 (46.4%) | 45 (53.6%) | |
| *CD8⁺ T-lymphocyte* | | | | | | | | | |
| Lowest tertile | | | | 55 (76.4%) | 17 (23.6%) | **<0.001** | 53 (75.7%) | 17 (24.3%) | **<0.001** |
| All others | | | | 58 (42.0%) | 80 (58.0%) | | 47 (32.9%) | 96 (67.1%) | |
| *CD45R0⁺ T- lymphocyte* | | | | | | | | | |
| Absent | | | | | | | 66 (70.2%) | 41 (37.6%) | **<0.001** |
| Present | | | | | | | 28 (29.8%) | 68 (62.4%) | |
| *CD8⁺/FoxP3⁺ ratio* | | | | | | | | | |
| Bottom tertile | | | | 41 (63.1%) | 24 (26.9%) | **0.038** | | | |
| All others | | | | 64 (47.4%) | 71 (52.6%) | | | | |
| *CD8⁺/CD45R0⁺ ratio* | | | | | | | | | |
| Bottom tertile | | | | | | | 50 (64.1%) | 28 (35.9%) | **<0.001** |
| All others | | | | | | | 43 (35.5%) | 78 (64.5%) | |

FIGO = International Federation of Gynaecology and Obstetrics. [a] P-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

**Table 3** Relationship of tumour-infiltrating lymphocytes in omental tissue to clinicopathological parameters and each other (N, %).

| | CD8⁺ T-lymphocyte | | | CD45R0⁺ T-lymphocyte | | | FoxP3⁺ T-lymphocyte | | |
|---|---|---|---|---|---|---|---|---|---|
| | lowest tertile | all others | p-value[a] | absent | present | p-value[a] | lowest tertile | all others | p-value[a] |
| *Age (years)* | | | | | | | | | |
| <59 | 28 (38.4%) | 45 (61.6%) | 0.173 | 38 (53.5%) | 33 (46.5%) | 0.495 | 28 (39.4%) | 43 (60.6%) | 0.653 |
| ≥59 | 16 (27.1%) | 43 (72.9%) | | 30 (47.6%) | 33 (52.4%) | | 21 (35.6%) | 38 (64.4%) | |
| *Tumour type* | | | | | | | | | |
| Serous | 32 (33.3%) | 64 (66.7%) | 1.000 | 52 (54.7%) | 43 (45.3%) | 0.149 | 38 (40.4%) | 56 (59.6%) | 0.299 |
| Non-serous | 12 (33.3%) | 24 (66.7%) | | 16 (41.0%) | 23 (59.0%) | | 11 (30.6%) | 25 (69.4%) | |
| *Differentiation grade* | | | | | | | | | |
| Grade I/II | 12 (41.4%) | 17 (58.6%) | 0.244 | 15 (51.7%) | 14 (48.3%) | 0.871 | 12 (42.9%) | 16 (57.1%) | 0.356 |
| Grade III & undiff. | 28 (29.8%) | 66 (70.2%) | | 47 (50.0%) | 47 (50.0%) | | 31 (33.3%) | 62 (66.7%) | |
| *Residual disease* | | | | | | | | | |
| <2cm | 7 (18.9%) | 30 (81.1%) | **0.035** | 18 (46.2%) | 21 (53.8%) | 0.488 | 8 (22.2%) | 28 (77.8%) | **0.028** |
| ≥2cm | 34 (38.2%) | 55 (61.8%) | | 47 (52.8%) | 42 (47.2%) | | 38 (43.2%) | 50 (56.8%) | |
| *CD8⁺ T-lymphocyte* | | | | | | | | | |
| Lowest tertile | | | | 35 (79.5%) | 9 (20.5%) | **<0.001** | 29 (59.2%) | 14 (17.5%) | **<0.001** |
| All others | | | | 29 (34.5%) | 55 (65.5%) | | 20 (40.8%) | 66 (82.5%) | |
| *CD45R0⁺ T- lymphocyte* | | | | | | | | | |
| Absent | | | | | | | 36 (73.5) | 27 (34.6%) | **<0.001** |
| Present | | | | | | | 13 (26.5%) | 51 (65.4%) | |
| *CD8⁺/FoxP3⁺ ratio* | | | | | | | | | |
| Bottom tertile | | | | 28 (66.7%) | 14 (33.3%) | **0.007** | | | |
| All others | | | | 35 (41.2%) | 50 (58.8%) | | | | |
| *CD8⁺/CD45R0⁺ ratio* | | | | | | | 34 (59.6%) | 23 (40.4%) | **<0.001** |
| Bottom tertile | | | | | | | 22 (27.5%) | 58 (72.5%) | |
| All others | | | | | | | | | |

FIGO = International Federation of Gynaecology and Obstetrics. [a] P-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

**Table 4** Multivariate Cox regression analysis on disease-specific survival.

| **All stages** | **H.R.** | **95% CI** | **p-value** | **H.R.** | **95% CI** | **p-value** |
|---|---|---|---|---|---|---|
| Age ≥ 59 years | 0.96 | 0.60-1.55 | 0.876 | 0.98 | 0.61-1.59 | 0.938 |
| FIGO Stage III/IV | 3.31 | 1.34-8.13 | **0.009** | 3.59 | 1.73-9.37 | **0.009** |
| Grade III/undifferentiated | 1.97 | 1.19-3.27 | **0.008** | 1.64 | 0.98-2.74 | 0.059 |
| Non-serous tumour | 0.67 | 0.41-1.12 | 0.125 | 0.63 | 0.38-1.05 | 0.078 |
| Residual tumour | 3.38 | 2.27-6.44 | **<0.001** | 3.01 | 1.82-4.96 | **<0.001** |
| $CD8^+$ T-lymphocytes high (OV) | 0.36 | 0.22-0.59 | **<0.001** | | | |
| $CD8^+/FoxP3^+$ ratio high (OV) | | | | 0.53 | 0.32-0.85 | **0.009** |

| **Advanced stage only** | **H.R.** | **95% CI** | **p-value** | **H.R.** | **95% CI** | **p-value** | **H.R.** | **95% CI** | **p-value** |
|---|---|---|---|---|---|---|---|---|---|
| Age ≥ 59 years | 0.82 | 0.50-1.36 | 0.444 | 1.02 | 0.63-1.64 | 0.945 | 0.99 | 0.60-1.64 | 0.979 |
| Grade III/undifferentiated | 1.75 | 1.02-2.99 | **0.041** | 1.82 | 1.07-3.09 | **0.026** | 1.69 | 0.96-2.97 | 0.067 |
| Non-serous tumour | 0.76 | 0.44-1.30 | 0.309 | 0.77 | 0.44-1.33 | 0.346 | 0.72 | 0.42-1.23 | 0.229 |
| Residual tumour | 3.58 | 2.08-6.17 | **<0.001** | 2.41 | 1.50-3.88 | **<0.001** | 2.56 | 1.54-4.25 | **<0.001** |
| $CD8^+$ T-lymphocytes high (OV) | 0.35 | 0.21-0.60 | **<0.001** | | | | | | |
| $CD45RO^+$ T-lymphocytes present (OV) | | | | 0.72 | 0.46-1.14 | 0.166 | | | |
| $FoxP3^+$ T-lymphocytes present (OV) | | | | | | | 0.55 | 0.34-0.88 | **0.013** |

| **Advanced stage only,** *cont.* | **H.R.** | **95% CI** | **p-value** | **H.R.** | **95% CI** | **p-value** | **H.R.** | **95% CI** | **p-value** |
|---|---|---|---|---|---|---|---|---|---|
| Age ≥ 59 years | 1.00 | 0.61-1.65 | 1.000 | 0.76 | 0.43-1.33 | 0.888 | 0.76 | 0.44-1.33 | 0.335 |
| Grade III/undifferentiated | 1.42 | 0.83-2.44 | 0.205 | 1.04 | 0.57-1.89 | 0.910 | 1.29 | 0.70-2.37 | 0.417 |
| Non-serous tumour | 0.76 | 0.44-1.30 | 0.318 | 0.60 | 0.34-1.06 | 0.078 | 0.66 | 0.37-1.20 | 0.177 |
| Residual tumour | 2.50 | 1.51-4.15 | **<0.001** | 2.63 | 1.41-4.90 | **0.002** | 2.74 | 1.46-5.15 | **0.002** |
| $CD8^+/FoxP3^+$ ratio high (OV) | 0.68 | 0.42-1.08 | 0.099 | | | | | | |
| $CD45RO^+$ T-lymphocytes present (OM) | | | | 0.69 | 0.43-1.08 | 0.106 | | | |
| $FoxP3^+$ T-lymphocytes high (OM) | | | | | | | 0.64 | 0.39-1.06 | 0.082 |

Analyses were performed stratified for type of chemotherapy. FIGO = International Federation of Gynecology and Obstetrics, H.R. = hazard ratio; C.I. = confidence interval; OV = ovarian-derived tumour tissue; OM = omental metastasis; Bold signifies p<0.05.

## DISCUSSION

The observation of an improved survival of patients with intra-tumoural T-lymphocytes suggests an important role for the immune system in the natural course of ovarian cancer (9). In the present study, we analyzed the relative contribution of different tumour-infiltrating T-lymphocyte subtypes to this observed improvement of survival. To our knowledge, this is the first study to examine the prognostic influence of different tumour-infiltrating T-lymphocytes in primary as well as metastatic lesions. We show improved disease-specific survival in advanced stage ovarian cancer patients with $CD45R0^+$ memory T-lymphocytes and/or $FoxP3^+$ regulatory T-lymphocytes infiltrating ovarian-derived tumour tissue and/or omental metastases. In addition, we also confirm important previous observations by others of improved survival in patients with either a high number of tumour-infiltrating $CD8^+$ cytotoxic T-lymphocytes or a high $CD8^+/FoxP3^+$ ratio (7).

Intra-tumoural $CD45R0^+$ T-lymphocytes, present in approximately 50% of patients, were associated with increased DSS when present in ovarian-derived tumour tissue and omental metastases. Although its expression is not restricted to memory T-lymphocytes, CD45R0 is commonly used as a marker for memory T-lymphocytes (5;17;24). Upon antigenic stimulation, naïve T-lymphocytes differentiate into effector T-lymphocytes, but also memory T-lymphocytes can be formed. The latter assure a strong and speedy immune response on subsequent exposures to the same antigen. The presence of tissue-infiltrating memory T-lymphocytes thus implies prior exposure to an antigen encountered in the tissue environment, which in case of tumour-infiltrating memory T-lymphocytes may very well reflect an anti-tumour response of the immune system. The presence of a survival benefit for patients with high levels of infiltrating memory T-lymphocytes was previously described in colorectal cancer (5;17). The cell numbers seen in these studies were remarkably higher than those used in our study (cut-off values 80-250 cells/$mm^2$), which may be explained in several ways. Firstly, no distinction was made between tumour- and peri-tumoural stroma infiltrating cells in these studies, whereas we only report tumour-infiltrating memory T-lymphocytes. Secondly, when repeating our analysis on the total cell count/core we still did not observe levels similar to that in colorectal cancer, although results for the survival analysis were comparable to the analysis with tumour-infiltrating memory T-lymphocytes only (data not shown). A more likely explanation is that the higher number of infiltrating lymphocytes in colon tissue could be considered inherent to its daily exposure to foreign products and pathogens, an observation which does not hold for ovarian tissue.

Our data show that patients with intra-tumoural CTL infiltrations and/or a high CTL/Treg ratio in tumour-containing ovarian tissue consistently have the longest disease-specific survival, confirming the results previously described in a smaller population of ovarian cancer patients (7). However, unlike previous studies in ovarian cancer addressing the prognostic influence of Treg (7;15), we found tumour-infiltrating Treg to be an independent positive factor for disease-specific survival in patients with advanced stage disease. One possible explanation is the fact that although FoxP3 is currently the best marker available for immunohistochemical staining of Treg on paraffin embedded tissue, it is not exclusively expressed in regulatory cells. Recently, it was shown that activated $CD4^+CD25^-$ effector T cells

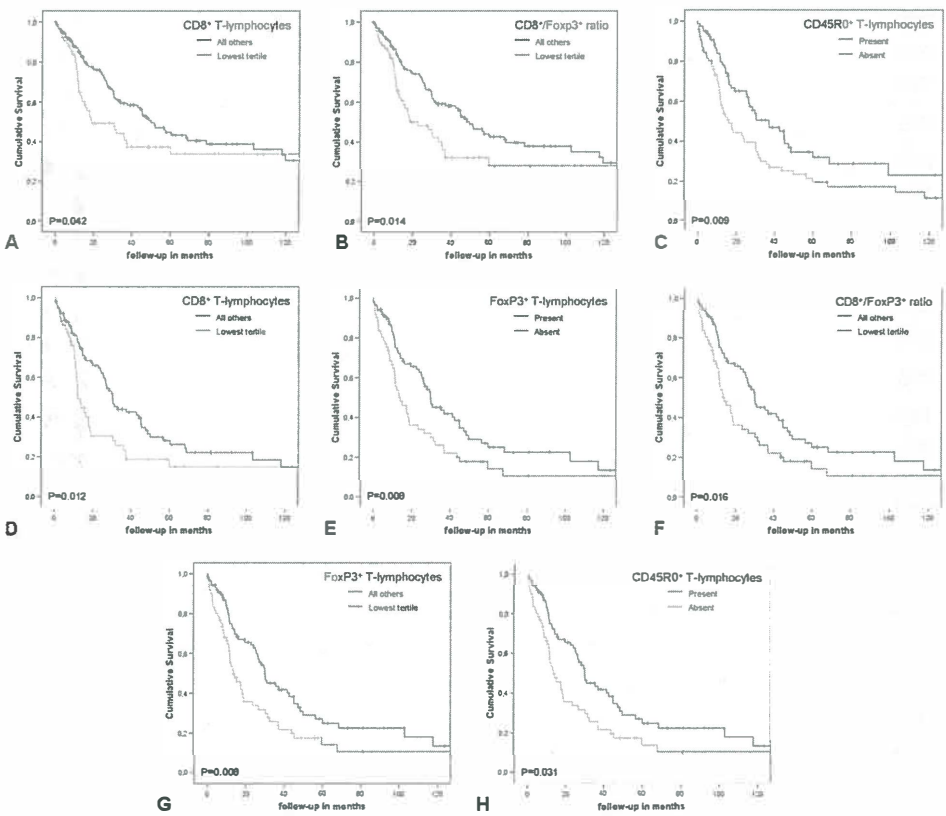


**Fig. 2** Disease-specific survival (in months) of ovarian cancer patients based on tumor-infiltrating lymphocytes. Cumulative survival time was estimated by Kaplan-Meier method. Log Rank test was used to evaluate survival differences between groups. High numbers of $CD8^+$ T-lymphocytes (**A**) and a high $CD8^+/FoxP3^+$ ratio (**B**) in ovarian derived tumour tissue were associated with improved survival in FIGO stage I-IV disease. In advanced stage patients, as survival benefit was observed for patients with $CD45RO^+$ T-lymphocytes (**C**) $CD8^+$ T-lymphocytes (**D**), $FoxP3^+$ T-lymphocytes (**E**), a high $CD8^+/FoxP3^+$ ratio (**F**) in ovarian-derived tumour tissue. Furthermore, $FoxP3^+$ (**G**) and $CD45RO^+$ T-lymphocytes (**H**) in omental metastases were associated with improved disease-specific survival.

transiently express FoxP3, without having regulatory activity (25;26). Possibly, the positive prognostic effect of the presence of $FoxP3^+$ cells in our samples can be attributed to the staining of not only suppressive, but also activated T-lymphocytes expressing FoxP3. Similar observations of positive effects of Treg have however previously been reported in studies on immune cell malignancies and head and neck squamous cell carcinoma (HNSCC), some of which further phenotyped the $FoxP3^+$ cells. A high number of Treg is associated to longer survival in follicular lymphoma and classic Hodgkin's lymphoma (27;28). In HNSCC where higher numbers of circulating Treg were present in patients without evidence of disease after primary treatment compared to patients with active disease, loco-regional Treg were associated with better loco-regional control (29;30). Furthermore, the possible protective role for Treg against cancer was also illustrated in murine models of colorectal cancer, which show that adoptive transfer of $CD4^+CD25^+$ regulatory cells results in regression of adenomas (31;32). Although we find that the presence of Treg is associated with an increase in disease-specific survival, we also find that 1) the absolute number of Treg present does not seem to alter this positive prognostic effect, 2) the presence of Treg is strongly associated with high numbers of CTL, and 3) a high CTL/Treg ratio is associated with a longer disease-specific survival. It thus seems plausible that it is not merely the presence of Treg, but rather the infiltration of tumour by T-lymphocytes in general which is responsible for the observed survival advantage, as was previously reported by Zhang et al. (9). In this point of view, infiltration of tumour epithelium, or any other inflammatory site, by lymphocytes is naturally accompanied by regulatory T-cells and the effectiveness of immune responses depends on the proportion of the different lymphocyte subtypes present instead of on the presence of a particular subtype.

Unlike previous studies of TIL in ovarian cancer, we have also studied the prognostic influence of TIL in omental metastases of ovarian cancer patients. Although TIL in omental metastases were not independent predictors of disease-specific survival, we did show an increased survival in univariate analysis for advanced stage patients with memory T-lymphocytes and Treg infiltrating omental metastases. Interestingly, median numbers of TIL were higher in omental metastases than in ovarian-derived tumour tissue and the percentage of patients with Treg in omental metastases was substantially higher than for ovarian-derived tumour tissue. The omentum may be considered a secondary lymphoid tissue as it contains large aggregates of macrophages and lymphocytes, the so-called milky spots. These milky spots contain mostly macrophages (68%), but T-lymphocytes are also present (10%) (33). The high percentage of patients with Treg observed in omental metastases and the higher median cell counts as compared to ovarian-derived tumour tissue may be a reflection of the lymphoid function of the omentum.

In summary, we show that memory T-lymphocytes infiltrating ovarian-derived tumour tissue and omental metastases are associated with increased disease specific survival in patients with advanced stage disease. Unexpectedly, the presence of regulatory T-lymphocytes in ovarian-derived tumour tissue is also an independent factor for increased disease specific survival. However, the fact that a high CTL/Treg ratio independently predicts increased survival as well, suggests that it is not so much the presence of Treg as the presence of TIL in general that is responsible for the observed survival effect. Our results support the notion that different T-lymphocyte subsets play a unique and important role in the natural course of ovarian cancer.

## REFERENCES


(1) Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001 Jan;51(1):15-36.

(2) Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005 Mar;55(2):74-108.

(3) Agarwal R, Linch M, Kaye SB. Novel therapeutic agents in ovarian cancer. Eur J Surg Oncol 2006 May 13;32(8):875-86.

(4) Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996 Apr 1;77(7):1303-10.

(5) Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006 Sep 29;313(5795):1960-4.

(6) Halpern AC, Schuchter LM. Prognostic models in melanoma. Semin Oncol 1997 Feb;24(1 Suppl 4):S2-S7.

(7) Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005 Dec 20;102(51):18538-43.

(8) Vesalainen S, Lipponen P, Talja M, Syrjanen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994;30A(12):1797-803.

(9) Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003 Jan 16;348(3):203-13.

(10) Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 2004 Jul 1;10(13):4450-6.

(11) Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998 Aug 15;58(16):3491-4.

(12) Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res 2001 Jul 1;61(13):5132-6.

(13) Schumacher K, Haensch W, Roefzaad C, Schlag PM. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 2001 May 15;61(10):3932-6.

(14) Yu P, Fu YX. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest 2006 Mar;86(3):231-45.

(15) Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004 Sep;10(9):942-9.

(16) Wolf D, Wolf AM, Rumpold H, Fiegl H, Zeimet AG, Muller-Holzner E, et al. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. Clin Cancer Res 2005 Dec 1;11(23):8326-31.

(17) Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005 Dec 22;353(25):2654-66.

(18) Cai XY, Gao Q, Qiu SJ, Ye SL, Wu ZQ, Fan J, et al. Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. J Cancer Res Clin Oncol 2006 May;132(5):293-301.

(19) Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.

(20) Scully RE. Histological Typing of Ovarian Tumours. 2 ed. Berlin: Springer; 1999.

(21) World Health Organization. Handbook for Reporting Results of Cancer Treatment. Geneva: World Health Organization; 1979.

(22) Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998 Jul;4(7):844-7.

(23) Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol 2004 Jul;17(7):790-7.

(24) Anraku M, Cunningham K, Yun Z, Tsao M, Zhang L, Keshavjee S, et al. Impact of tumor-infiltrating T cells on survival in patients with malignant pleural mesothelioma. The Journal of Thoracic and Cardiovascular Surgery 2008 Apr;135(4):823-9.

(25) Allan S, Crome S, Crellin N, Passerini L, Steiner T, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007 Apr 1;19(4):345-54.

(26) Roncarolo MG, Gregori S. Is FOXP3 a bona fide marker for human regulatory T cells? Eur J Immunol 2008 Apr;38(4):925-7.

(27) Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J, et al. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 2005 Feb 15;11(4):1467-73.

(28) Lee A, Clear A, Calaminici M, Davies A, Jordan S, MacDougall F, et al. Number of CD4+ Cells and Location of Forkhead Box Protein P3-Positive Cells in Diagnostic Follicular Lymphoma Tissue Microarrays Correlates With Outcome. J Clin Oncol 2006 Nov 1;24(31):5052-9.

(29) Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NH, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res 2006 Jan 15;12(2):465-72.

(30) Strauss L, Bergmann C, Gooding W, Johnson J, Whiteside T. The Frequency and Suppressor Function of CD4+CD25highFoxp3+ T Cells in the Circulation of Patients with Squamous Cell Carcinoma of the Head and Neck. Clin Cancer Res 2007 Nov 1;13(21):6301-11.

(31) Erdman S, Poutahidis T, Tomczak M, Rogers A, Cormier K, Plank B, et al. CD4+ CD25+ Regulatory T Lymphocytes Inhibit Microbially Induced Colon Cancer in Rag2-Deficient Mice. Am J Pathol 2003 Feb 1;162(2):691-702.

(32) Erdman S, Sohn J, Rao V, Nambiar P, Ge Z, Fox J, et al. CD4+CD25+ Regulatory Lymphocytes Induce Regression of Intestinal Tumors in ApcMin/+ Mice. Cancer Res 2005 May 15;65(10):3998-4004.

(33) Krist LF, Eestermans IL, Steenbergen JJ, Hoefsmit EC, Cuesta MA, Meyer S, et al. Cellular composition of milky spots in the human greater omentum: an immunochemical and ultrastructural study. Anat Rec 1995 Feb;241(2):163-74.

# Chapter 3

## Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation



Leffers N, Lambeck A, de Graeff P, Bijlsma A, Daemen T, van der Zee A, Nijman H

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## ABSTRACT

**Objectives**

The adaptive immune system seems to play an essential role in the natural course of ovarian cancer. Aim of this study was to establish whether disease-specific survival for patients expressing the tumour antigen p53 is influenced by MHC class I expression or the presence of p53-autoantibodies (p53-Aab).

**Methods**

P53 and MHC class I expression were analysed in ovarian cancer tissue of 329 patients by immunohistochemistry using tissue microarrays. For 233 patients, pre-treatment serum samples were available to study the presence of p53-autoantibodies by ELISA. Data were linked to clinicopathological parameters and disease-specific survival.

**Results**

P53-overexpression, MHC class I down-regulation in neoplastic cells and serum p53-autoantibodies were observed in 49.4, 38.9 and 15.9% of patients, respectively. MHC class I down-regulation in p53-overexpressing tumours correlated with a 10-month reduced disease-specific survival in univariate analysis (log-rank 4.10; $p=0.043$). p53-Aab were strongly correlated with p53-overexpression ($p<0.001$), but did not influence disease-specific survival.

**Conclusions**

As the prognosis of patients with p53-overexpressing ovarian cancer is affected by the MHC class I status of tumour cells and ovarian cancer patients can generate immune responses to the p53 tumour antigen, the further development of immunotherapy should evaluate strategies to improve MHC class I expression by tumour cells to facilitate antigen presentation in an attempt to increase clinical responses.

## INTRODUCTION

Ovarian cancer is the most frequent cause of death due to gynaecological malignancies. As a result of the absence of specific symptoms, 70% of patients are diagnosed with advanced stage disease. Despite standard treatment, i.e. cytoreductive surgery and platinum-based chemotherapy, almost all patients with advanced stage disease at presentation will relapse, resulting in a median progression-free survival of only 18 months. Advances in (chemo)therapeutic strategies in the last decades have not substantially altered five-year survival (1;2). Immunotherapy is one of the novel therapeutic strategies under investigation in ovarian cancer.

All human body cells present antigens to the immune system by means of major histocompatibility complex (MHC) class I molecules on the cell surface, which can be recognised by cytotoxic T-lymphocytes (CTL). In principle, only antigen-presenting cells, such as dendritic cells, possess both MHC class I and class II molecules and co-stimulatory molecules, involved in the induction and activation of CTL as well as T helper and B-lymphocytes. The majority of antigens presented by human body cells, will not be recognised by CTL due to the deletion of immature T-lymphocytes with high affinity for self-antigens in the thymus. Like normal human body cells, tumour cells express antigens to the immune system by means of MHC class I molecules. MHC restricted (tumour) antigens can be recognised as non-self or altered, and thus allow for the differential recognition and destruction of tumour cells by the immune system. Increased survival observed in ovarian cancer patients with intra-tumoural T-lymphocytes in tumour tissue suggests an important role for the immune system in the natural course of this disease (3).

A tumour antigen of interest in ovarian cancer is the p53 protein. This tumour-suppressor protein causes cell cycle arrest in case of DNA damage to allow DNA repair or, in case of substantial damage, induction of apoptosis. P53 is overexpressed in 50-60% of ovarian cancers (4-8), which is due to mutations in the p53 gene in 50% of tumours (9). The different p53 expression levels between healthy and tumour cells, in combination with possible differential handling by the proteasome complex of the p53 protein in tumour versus normal cells, might allow for p53-specific immunity in cancer patients. A schematic presentation of the dependence of the p53 tumour antigen on MHC class I and class II molecules to induce cellular and humoural p53-specific immune responses is depicted in figure 1. Evidence for both p53-specific cellular and humoural immunity in ovarian cancer patients is available. We recently demonstrated the presence of a weak p53-specific T-lymphocyte repertoire in 50% of patients with ovarian cancer (10). In addition, p53-specific auto-antibodies (p53-Aab) appear to be present in 18-25% of ovarian cancer patients (11-15). Until lately, the presence of the p53-Aab was generally believed, but not always found to be a predictor of unfavourable prognosis (11;13;15). In contrast, Goodell et al. (14) recently reported the presence of p53-Aab to be an independent

variable for improved overall survival in advanced stage ovarian cancer patients. The discussion on the influence of p53-specific humoural immunity on prognosis is thus still unresolved. Another important issue in tumour immunology is a phenomenon known as immunoselection, i.e. tumour cells with less immunogenic characteristics have a survival benefit over tumour cells that are easily recognised by the immune system (16;17). A key example of immunoselection is down-regulation of MHC class I molecules on the cell surface, which prevents recognition of tumour cells by CTL. Down-regulation of MHC class I has been described in several malignancies, including carcinomas of the colon, breast, lung, kidney, cervix and prostate (18-23). In colon and lung cancer, down-regulation of MHC class I was associated with advanced stage of disease and poor survival (18;23). For ovarian cancer, MHC class I down-regulation has been observed in approximately 35% of patients (24;25). In these studies no association of MHC class I down-regulation was found with survival (24;25).

We hypothesised that 1) disease-specific survival for patients with MHC class I down-regulation in the presence of a tumour antigen (p53-overexpression), would be shorter compared to patients with normal MHC class I expression, and 2) the presence of p53-Aab would not be independently associated with survival as antibodies are not effective in the attack of intracellular proteins.

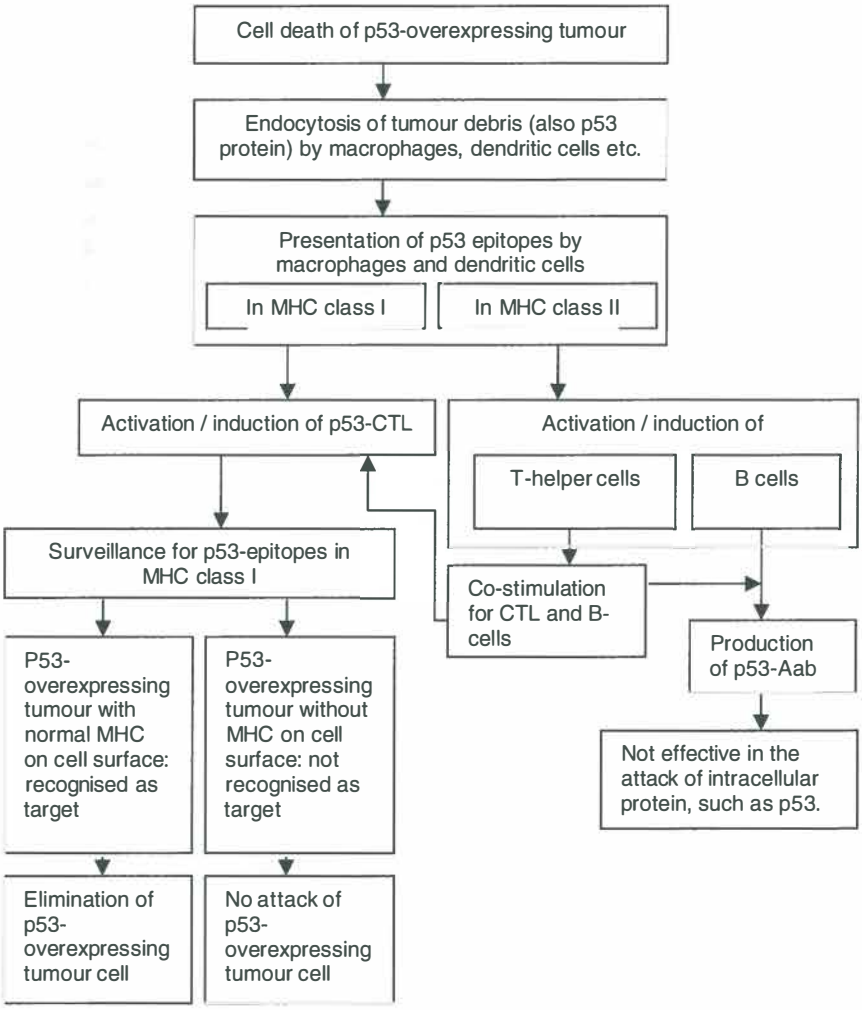


**Fig. 1** Schematic presentation of the dependence of the p53 tumour antigen on MHC class I and II to induce cellular and humoural p53-specific immune responses

## MATERIALS AND METHODS

### Patients

Since 1985 the Department of Gynaecological Oncology at the University Medical Center Groningen (UMCG) keeps a computerised database of epithelial ovarian cancer patients treated by gynaecological oncologists from this hospital at any time point during the course of their disease, prospectively collecting information on clinicopathologic characteristics and follow-up. In addition, since 1974 serum from patients with ovarian cancer obtained at time of diagnosis and different time points during the course of disease and treatment is stored at -80°C in a serum bank.



For this study, ovarian cancer patients were selected if they underwent primary surgery between May 1985 and June 2006 and if paraffin-embedded tumour tissue was available.

Primary treatment generally consisted of surgery, which entails total abdominal hysterectomy, omentectomy, multiple peritoneal and lymph node samplings as well as peritoneal washings for cytology. Patients were staged according to FIGO classification (26). Tumours were graded and classified according to WHO criteria by a gynaecological pathologist (27). Adjuvant chemotherapy consisted of different platinum-based treatment regimens. After treatment, patients were followed-up for at least 10 years with gradually increasing intervals. Follow-up data were completed for all patients until January 2007.

### Institutional Review Board Approval

For the present study, all relevant data were retrieved from our computerized database into a separate anonymous database. In this separate, password-protected database, patient identity was protected by study-specific, unique patient codes, which were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathological and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Based on this information, according to Dutch law no further approval from our IRB was needed.

### Tissue microarrays

Tissue microarrays (TMA) were constructed as described in previous studies (28;29). In brief, paraffin-embedded tissue blocks and corresponding haematoxylin & eosin (H&E) stained slides were retrieved from the pathology archives. Representative areas of tumour, derived from different anatomical locations, were marked on the respective H&E stained slides. Next, four 0.6mm core biopsies were taken from each tumour specimen and arrayed on a recipient paraffin block using a tissue microarrayer (Beecher instruments, Silver Spring, Maryland, USA). Adhesion of cores to the recipient block was accomplished by placing the blocks in a 37°C oven for fifteen minutes. For staining, 4µm sections were cut from each TMA block and

applied to APES-coated slides. H&E staining was performed to verify the presence of tumour in the arrayed samples.

**Immunohistochemical staining of TMAs**

TMA sections were stained with a p53-wildtype and p53-mutation recognising monoclonal antibody, clone DO-7 (Dako Netherlands BV, Heverlee, Belgium), and a HLA-type I heavy chain specific monoclonal antibody, clone HC10 (kind gift from Prof. J. Neefjes, NKI, Amsterdam, The Netherlands). In brief, TMA sections were dewaxed in xylene and rehydrated using graded concentrations of ethanol to distilled water. Antigen retrieval was performed by boiling TMA sections in 10mMTris/1mM EDTA at pH 9.0 for 15 min in a microwave oven. Endogenous peroxidase activity was blocked by submersion of sections in a 0.3% $H_2O_2$ solution for 30 min. Sections were incubated with the primary antibody (dilutions: DO-7 1:1000; HC10 1:500) for 60 min. Sections stained with DO-7 were subsequently incubated with DAKO Envision+ for 30 min. For sections stained with HC10, $RAM^{po}$ (dilution 1:100) and $GAR^{po}$ (dilution 1:100) were used as secondary and tertiary antibodies respectively. The antigen-antibody reactions were visualised with 3,3'-diaminobenzidine. Sections were counterstained with haematoxylin, dehydrated in graded concentrations of ethanol, dried and mounted.

**Scoring**

P53 staining for all TMAs was scored independently by two observers (P.G., A.B.). HLA type I heavy chain was scored independently by A.B. and N.L. Observers had no prior knowledge of clinicopathological information. To obtain a high concordance rate with whole tissue slides, it was decided that minimally two cores with representative tumour tissue had to be present on the TMA for a sample to be entered into analysis (29).

For DO-7, immunoreactivity was scored according to intensity of nuclear staining and percentage of positively stained tumour cells. Tumours showing >50% immunostaining with moderate or strong intensity were considered as having aberrant p53-immunostaining, as previously published (5). This cut-off value was based on the observation of weakly or moderately positive immunostaining in normal control tissues.

For HC10, immunoreactivity was scored according to the percentage of positively stained tumour cells and the intensity of membrane staining compared to stromal and/or endothelial staining. Tumours showing <25% immunostaining with weak intensity were considered to have HLA type I heavy chain down-regulation, as previously published by Vitale et al (24).

**ELISA**

Availability of serum samples was checked for all patients on the TMA. The presence of p53-autoantibody was determined by using a commercially available ELISA kit according to manufacturer's instructions (Dianova, Hamburg, Germany). In brief,

serum samples were diluted in reconstituted sample dilution buffer at a concentration of 1:100. A calibrator solution (human serum with a defined p53 autoantibody titer) was added to each microtiter plate in duplo at six different dilutions: undiluted, 1:1.5, 1:2, 1:3, 1:4 1:6, corresponding to a titer of 1, 0.67, 0.5, 0.33, 0.25 and 0.16 Units respectively. Microtiter plates were hydrated with 200µl washing solution per well for 3 minutes. After draining of residual fluid, calibrator and negative controls as well as diluted serum samples were added at 100µl per well and incubated at room temperature for 1 hour. Serum samples were randomly distributed over microtiter plates. Next, plates were washed 5 times with washing solution. Detector antibody was added at 100µl per well, left to incubate at room temperature for 1 hour. Microtiter plates were then washed 5 times with washing solution. Substrate solution was added at 100µl per well and incubated in the dark at room temperature for 30 minutes, after which 50µl of stop solution was added per well to stop the enzymatic reaction. Absorption was read at 450nm. Based on the measured absorption of the six calibration samples, a calibration curve was constructed with absorption on the y-axis and the calculated titer on the x-axis for each microtiter plate. The cut-off for each microtiter plate was calculated according to the manufacturer's instruction as the absorption of the undiluted calibrator x 0,15. Samples with an absorption <20% above the cut-off were considered to lie within a critical range and needed to be retested. Positive samples and samples within the critical range were tested in duplo to confirm results.

**Statistical analysis of data**

Associations between clinicopathological characteristics and immunohistochemical staining patterns were tested using Pearson Chi square tests. This test was also used to test for the association between p53 expression and the presence of p53 auto-antibodies. To test whether serum storage time influences the detection of p53 auto-antibodies, a Mann-Whitney-U test was performed. Disease-specific survival (DSS) was plotted using Kaplan Meier and Log Rank test was used to assess survival differences between groups. Disease-specific survival was defined as date of surgery until death due to ovarian cancer or date of last follow-up. Cox proportional hazards model was used for multivariate analysis and was stratified for type of chemotherapy. Only variables with statistically significant associations in univariate analysis were used for multivariate analysis. For all tests, p-values <0.05 were considered significant. All p-values were two-sided. All statistical analyses were performed using SPSS 14.0 software package for windows (SPSS Inc., Chicago, IL, USA).

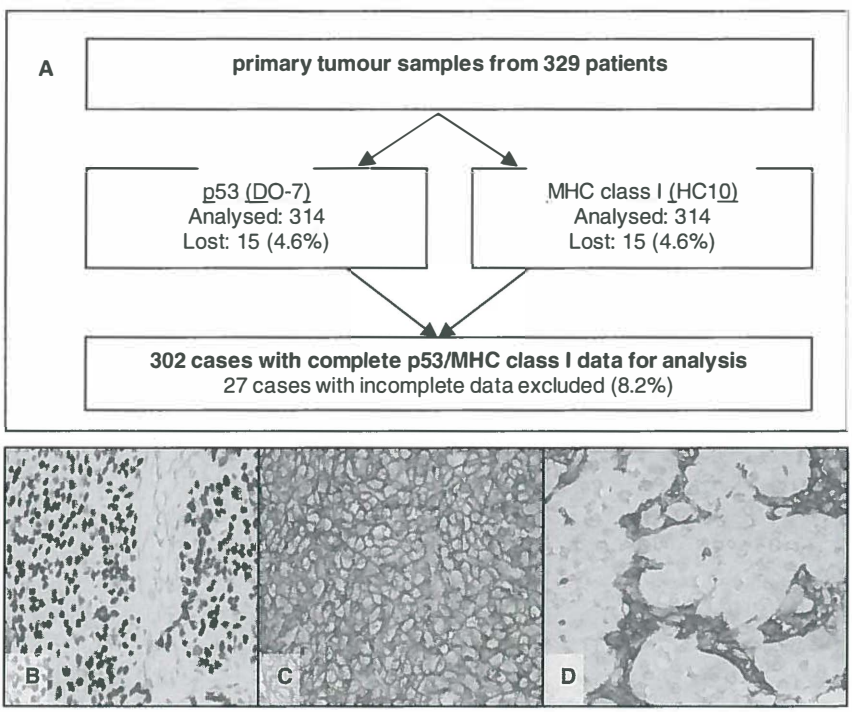


**Figure 2 Immunostaining of ovarian cancer for p53 and MHC class I** A. Flow diagram showing the origin of the subgroup analysed for both p53 and MHC class I expression. B. Strong nuclear p53 staining in ovarian cancer cells observed in 155/314 patients. C. Normal membrane staining of MHC class I in ovarian cancer cells. D. Down-regulation of membrane MHC class I expression in ovarian cancer cells observed in 122/314 patients (400x).

**Table 1** Clinicopathological characteristics and survival data

| | Pre-chemotherapy tumour samples (n=329) | |
|---|---|---|
| *Age (years)* | | |
| Median (range) | 59 | (16-89) |
| *Disease-specific survival (months)* | | |
| Median (95% CI) | 37.4 | (27.5-47.3) |
| *FIGO Stage* | | |
| Stage I | 69 | (21.0%) |
| Stage II | 26 | (7.9%) |
| Stage III | 184 | (55.9%) |
| Stage IV | 48 | (14.6%) |
| Missing | 2 | (0.6%) |
| *Tumour type* | | |
| Serous | 185 | (56.2%) |
| Mucinous | 37 | (11.2%) |
| Endometroid | 44 | (13.4%) |
| Clear Cell | 21 | (6.4%) |
| Adenocarcinoma | 15 | (4.6%) |
| Mixed Tumours | 17 | (5.2%) |
| Other | 10 | (3.0%) |
| *Tumour Grade* | | |
| Grade I | 52 | (15.8%) |
| Grade II | 89 | (27.1%) |
| Grade III | 145 | (44.1%) |
| Undifferentiated | 15 | (4.6%) |
| Missing | 28 | (8.5%) |
| *Residual disease* | | |
| < 2 cm | 168 | (51.1%) |
| >= 2 cm | 140 | (42.6%) |
| Missing | 21 | (6.4%) |
| *Type of chemotherapy* | | |
| No chemotherapy | 45 | (13.7%) |
| Platinum containing | 122 | (37.1%) |
| Platinum & taxane containing | 128 | (38.9%) |
| Other regimen | 28 | (8.5%) |
| Missing | 6 | (1.8%) |

CI = confidence interval; FIGO = International Federation of Gynaecology and Obstetrics.

## RESULTS

### Study population

For 329 ovarian cancer patients who underwent surgery by a gynaecological oncologist from the UMCG between May 1985 and June 2006, sufficient paraffin-embedded tumour tissue was available to be included in the study. Clinicopathological data are summarised in Table 1. Of the 45 patients not treated with chemotherapy, 29 were diagnosed with stage I disease. The remaining 16 patients were either not fit or unwilling to receive chemotherapy. Of the patients treated with chemotherapy 89.9% received a platinum-based regimen. Estimated five year disease-specific survival rates for the whole group, early stage and advanced stage disease were 44%, 83% and 29% respectively (data not shown). Median disease-specific survival was 37.4 months (95% C.I.: 27.5-47.3).

### Immunohistochemistry and ELISA

A total of 302 patients (91.8%) could be evaluated on TMA for both p53 and MHC class I expression (figure 2a). P53-overexpression was observed in pre-chemotherapy tumour samples of 155 (49.4%) patients (figure 2b). For 188 patients (62%), data on p53-overexpression were previously published (5) and comparable to the new, expanded group (47.3% vs. 49.4%, data not shown). MHC class I down-regulation was observed in 122 (38.9%) of patients (figure 2c/d). For 233 patients (70.8%) both pre-treatment serum samples and p53-expression data were available. Serum samples of thirty-seven (15.9%) patients were found to be positive for p53-Aab. The detection of p53-Aab was not associated with the time since storage in the serum bank (p=0.980).

Table 2 shows the associations between clinicopathological parameters, p53 expression, MHC class I expression and the presence serum of p53-Aab. P53-overexpression in tumour tissue was associated with a higher age at diagnosis (p=0.007), advanced stage disease (p<0.001), serous tumour type (p=0.008), poorly differentiated tumours (p=0.001) and ≥2 cm residual disease after primary debulking surgery (p=0.005). MHC class I down-regulation did not correlate with any well-known prognostic factor. Patients with p53-Aab more frequently had advanced stage disease (19.2% vs. 6.6%, p=0.012) and poorly differentiated tumours (19.3% vs. 6.8%, p=0.007). The presence of serum p53-Aab was strongly associated with p53-overexpression in the primary tumour (p<0.001).

### Survival analysis

Univariate analysis of disease-specific survival showed that p53-overexpression as well as MHC class I down-regulation were associated with a shorter disease-specific survival (p<0.001, p=0.037 respectively; figure 3a/b). Confirming our hypothesis, patients with p53-overexpression and MHC class I down-regulation (n=59) had a 10 month shorter median disease-specific survival compared to patients with p53-overexpression and normal MHC class I staining (n=92; 21.1 vs. 31.9 months,

p=0.043; figure 3c). There was no survival difference based on MHC class I expression for patients with normal p53-expression (p=0.179; figure 3d).
As hypothesised, in the subgroup for which serum was available, the presence of serum p53-Aab was not found to influence disease-specific survival (p=0.390; figure 3e). To allow for comparison of our results to the results of Goodell et al. (14), univariate survival analysis was repeated in a subgroup containing only advanced stage patients and again no prognostic effect of serum p53-Aab was observed (p=0.912, data not shown).



We performed subgroup analysis using the well-defined group of patients with a serous tumour. No such analysis was performed for the non-serous group because of its miscellaneous composition. Patients with a serous tumour showed a trend towards a decreased median disease-specific survival in case of p53-overexpression and MHC class I down-regulation (14.7 vs. 31.0 months, p=0.055; figure 3f), but no survival difference was seen observed on MHC class I expression in patients with normal p53-expression (p=0.996, data not show).

To assess the importance of the individual variables found to be associated with disease-specific survival in the univariate analysis, multivariate analyses were performed including clinicopathological parameters (Table 3). After stratification for type of chemotherapy, only advanced stage disease at presentation, serous tumour type and ≥2cm residual disease after primary debulking surgery were found to independently predict a poor prognosis. A multivariate analysis in the subgroup with p53-overexpression also did not show MHC class I expression to be an independent prognostic factor.

**Table 2** Relationship of p53 expression, MHC class I expression and serum p53-Aab to clinicopathological parameters and each other.

| | P53 expression | | | MHC class I expression | | | Serum p53-Aab | | |
|---|---|---|---|---|---|---|---|---|---|
| | normal | excessive | p-value[a] | normal | downregulation | p-value[a] | negative | positive | p-value[a] |
| *Age (years)* | | | | | | | | | |
| <59 | 92 (58.2%) | 66 (41.8%) | **0.007** | 104 (65.8%) | 54 (34.2%) | 0.087 | 108 (87.8%) | 15 (12.2%) | 0.202 |
| ≥59 | 67 (42.9%) | 89 (57.1%) | | 88 (56.4%) | 68 (43.6%) | | 90 (81.8%) | 20 (18.2%) | |
| *FIGO stage* | | | | | | | | | |
| Stage I/II | 65 (72.2%) | 25 (27.8%) | **<0.001** | 59 (67.8%) | 28 (32.2%) | 0.119 | 71 (93.4%) | 5 (6.6%) | **0.012** |
| Stage III/IV | 93 (41.9%) | 129 (58.1%) | | 131 (58.2%) | 94 (41.8%) | | 126 (80.8%) | 30 (19.2%) | |
| *Tumour type* | | | | | | | | | |
| Serous | 78 (44.1%) | 99 (55.9%) | **0.008** | 109 (61.6%) | 68 (38.4%) | 0.857 | 105 (84.0%) | 20 (16.0%) | 0.653 |
| Non-serous | 81 (59.1%) | 56 (40.9%) | | 83 (60.6%) | 54 (39.4%) | | 93 (86.1%) | 15 (13.9%) | |
| *Differentiation grade* | | | | | | | | | |
| Grade I/II | 83 (61.9%) | 51 (38.1%) | **0.001** | 80 (60.6%) | 52 (39.4%) | 0.619 | 96 (93.2%) | 7 (6.8%) | **0.007** |
| Grade III & undiff. | 65 (41.9%) | 90 (58.1%) | | 99 (63.5%) | 57 (36.5%) | | 92 (80.7%) | 22 (19.3%) | |
| *Residual disease* | | | | | | | | | |
| <2cm | 93 (58.5%) | 66 (41.5%) | **0.005** | 102 (65.0%) | 55 (35.0%) | 0.158 | 117 (88.6%) | 15 (11.4%) | 0.109 |
| ≥2cm | 57 (41.9%) | 79 (58.1%) | | 78 (56.9%) | 59 (43.1%) | | 72 (80.9%) | 17 (19.1%) | |
| *P53 expression* | | | | | | | | | |
| Normal | | | | 89 (58.9%) | 62 (41.4%) | 0.725 | 123(96.1%) | 5 (3.9%) | **<0.001** |
| Excessive | | | | 92 (60.9%) | 59 (39.1%) | | 75 (71.4%) | 30 (28.6%) | |
| *MHC class I expression* | | | | | | | | | |
| Normal | | | | | | | 118 (82.5%) | 25 (17.5%) | 0.418 |
| Excessive | | | | | | | 77 (86.5%) | 12 (13.5%) | |

Patients were entered into analysis when information on both clinicopathological parameter and serum p53-Aab , p53 or MHC class I tissue expression was available. FIGO = International Federation of Gynaecology and Obstetrics. [a] P-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

**Table 3** Multivariate Cox regression analysis on disease-specific survival.

| | All patients | | | | | | Subgroup with p53-overexpression | | |
|---|---|---|---|---|---|---|---|---|---|
| | *P* | HR | 95% CI | *P* | HR | 95% CI | *P* | HR | 95% CI |
| Age > 59 years | 0.880 | 0.97 | 0.66-1.44 | 0.824 | 0.96 | 0.64-1.42 | 0.744 | 0.90 | 0.49-1.66 |
| Stage III/IV | **0.001** | 4.23 | 1.78-10.01 | **0.001** | 4.46 | 1.89-10.57 | 0.128 | 2.31 | 0.79-6.81 |
| Non-serous tumour type | **0.032** | 0.62 | 0.40-0.96 | **0.040** | 0.63 | 0.41-0.98 | 0.307 | 0.72 | 0.39-1.35 |
| Grade III / undiff | 0.113 | 1.40 | 0.92-2.11 | 0.144 | 1.36 | 0.90-2.06 | 0.830 | 1.06 | 0.61-1.87 |
| Residual disease ≥ 2 cm | **<0.001** | 2.69 | 1.77-4.08 | **<0.001** | 2.57 | 1.70-3.89 | **0.002** | 2.56 | 1.14-4.66 |
| P53-overexpression | 0.802 | 1.05 | 0.73-1.50 | | | | | | |
| MHC class I down-regulation | | | | 0.73 | 1.07 | 0.73-1.57 | 0.672 | 1.13 | 0.65-1.97 |

Analyses were stratified for type of chemotherapy. HR = hazard ratio; CI = confidence interval; Bold signifies p-value <0.05.

## DISCUSSION & CONCLUSION

An improved survival of patients with intra-tumoural T-cells suggests an important role for the immune system in the natural course of ovarian cancer (3;30). A necessary requirement for a clinically relevant adaptive immune response is processing and presentation of tumour associated antigens to the immune system. In this large historical cohort study, we show a survival advantage of normal MHC class I expression compared to MHC class I down-regulation for patients with p53-overexpressing tumours, a well known tumour-antigen. In addition, we demonstrate that overexpression of p53 in tumours induces humoural p53-specific immunity in only a minority of ovarian cancer patients, while refuting the prognostic relevance of serum p53-Aab in ovarian cancer patients.



Unlike previously published studies in ovarian cancer that found no association with survival, we found MHC class I down-regulation to negatively influence survival in this large cohort (24;25). Moreover, confirming our hypothesis, analysis of the expression of MHC class I molecules in tumours of patients with p53-overexpressing tumours, showed a longer disease-specific survival for patients with normal MHC class I staining compared to MHC class I down-regulation. Our hypothesis was further confirmed by the absence of an association of disease-specific survival with MHC class I expression in tumours with normal p53-expression. The difference in survival seen in patients with overexpression of a specific tumour antigen based on MHC class I expression suggests a significant role for the immune system in tumour behaviour, as expression of MHC class I is a requirement for recognition and destruction of the tumour by tumour antigen specific CTL.

Our results may have important implications for antigen-specific active immunotherapy, which aims at tumour destruction through the induction of CTL directed towards a specific target antigen by administration of a molecular defined antigen-specific vaccine to the patient. Although many immunotherapeutic trials show induction of tumour-specific T-lymphocytes, clinical responses are often disappointing (e.g.(31-38)). MHC class I down-regulation, as one of the known tumour immune escape mechanisms (17), may partly explain these disappointing results, although this cannot be verified as information on MHC class I expression of tumours is not generally available for these studies. Selection of patients for immunotherapy based on MHC class I expression of tumours may be an approach to improve clinical responses to immunotherapy. Alternatively, combining immunisation with strategies to increase MHC class I expression may also be an option. For instance, in murine studies it has been shown that strategies to increase interferon-γ concentrations in the tumour micro-environment can cause repair of MHC class I defects (39;40).

Another promising approach is the administration of 5-aza-2'-deoxycytidine (DAC), a DNA demethylating agent, which has been shown to increase MHC class I expression levels of tumour cells (41). In addition, in a human study DAC was demonstrated to upregulate the expression of certain tumour antigens and proved to be well-tolerated (42). Co-administration of DAC with immunotherapy may thus increase the immunogenicity of tumour cells as well as increase the recognition of tumour cells by CTL.



We identified serum p53-Aab in 15% of ovarian cancer patients. The presence of serum p53-Aab was strongly associated with p53-overexpression and only five patients with p53-Aab did not have nuclear p53-overexpression. However, two of these five patients did show cytoplasmatic p53-staining (data not shown). In contrast to the recently published study by Goodell et al, the presence of serum p53-Aab was not associated with disease-specific survival in our study (14). Multivariate survival analysis in the study by Goodell et al. was performed in the advanced stage subgroup only, without taking into account the amount of residual disease, which is a well acknowledged and very strong prognostic factor (14). Finally, analysis of our data according to the statistical methods used by Goodell et al, did not change our results (14).

The apparent lack of efficacy of serum p53-Aab to influence prognosis by eradicating tumours does not come as a surprise, as antibodies are not capable of recognising intracellular or intranuclear proteins, such as the p53 protein which is not expressed on the cell surface of tumour cells. The strong association of serum p53-Aab with p53-overexpression in tumour tissue combined with the lack of prognostic value of serum p53-Aab thus suggests that circulating p53-Aab are indeed a by-product of aberrant p53-expression. However, from an immunological point of view, serum p53-Aab remain interesting, as B-lymphocytes are dependent on co-stimulation by T helper lymphocytes for the isotype switching of IgM to IgG antibodies (43). The presence of p53-Aab is therefore a reflection of the ability of the immune system to induce p53-specific T helper responses.

In conclusion, our current data indicate the prognostic value of MHC class I in ovarian cancer with p53-overexpression. To increase clinical responses, the combination of p53-based immunotherapeutic strategies with schemes to improve MHC class I expression by tumour cells could be explored.

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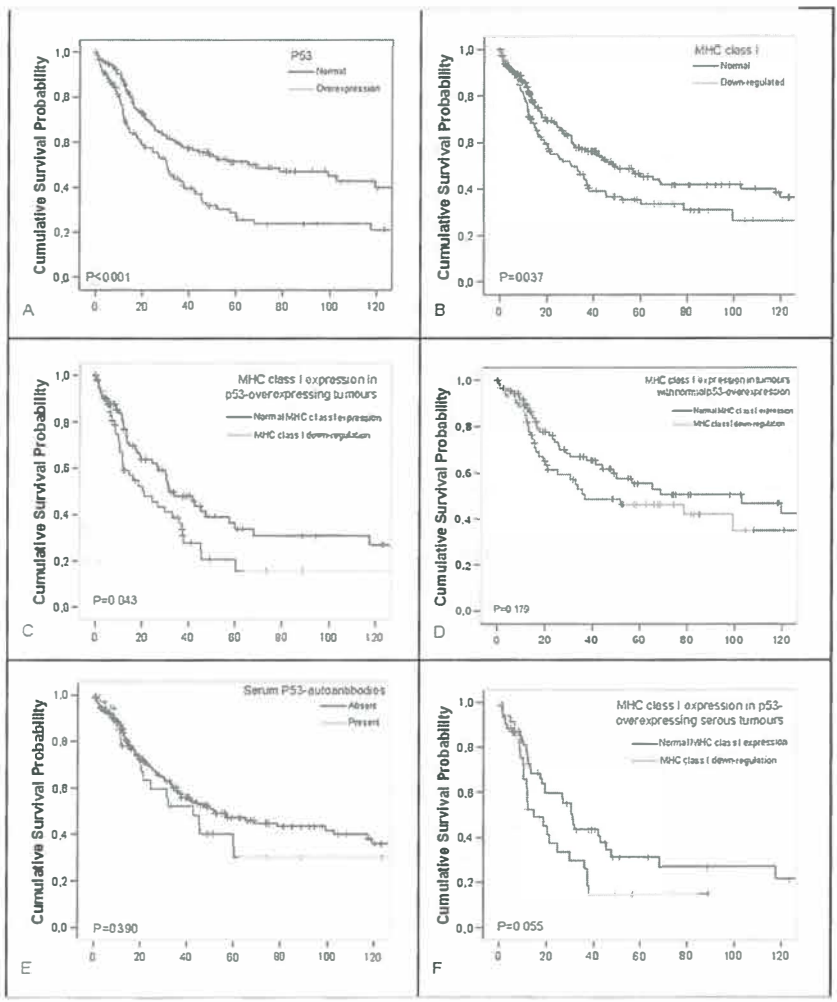


**Fig. 3** Kaplan-Meier analysis of disease-specific survival of ovarian cancer patients (in months). A. According to p53-staining. B. According to MHC-class I staining. C. According to MHC class I expression in p53-overexpressing tumours. D. According to MHC class I staining in normal p53 expressing tumours. E. According to serum p53-Aab status. F. According to MHC class I expression in p53-overexpressing serous tumours. Survival differences between groups were estimated using log-rank test.

## REFERENCES


(1) Agarwal R, Linch M, Kaye SB. Novel therapeutic agents in ovarian cancer. Eur J Surg Oncol 2006;32(8):875-86.

(2) Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.

(3) Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348(3):203-13.

(4) Nijman HW, Lambeck A, van der Burg SH, van der Zee AG, Daemen T. Immunologic aspect of ovarian cancer and p53 as tumor antigen. J Transl Med 2005;3:34.

(5) de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006;95(5):627-33.

(6) Klemi PJ, Pylkkanen L, Kiilholma P, Kurvinen K, Joensuu H. p53 protein detected by immunohistochemistry as a prognostic factor in patients with epithelial ovarian carcinoma. Cancer 1995;76(7):1201-8.

(7) Nielsen JS, Jakobsen E, HOlund B, Bertelsen K, Jakobsen A. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. International Journal of Gynecological Cancer 2004;14(6):1086-96.

(8) van der Zee AG, Hollema H, Suurmeijer AJ, Krans M, Sluiter WJ, Willemse PH, et al. Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 1995;13(1):70-8.

(9) Bartel F, Jung J, Bohnke A, Gradhand E, Zeng K, Thomssen C, et al. Both Germ Line and Somatic Genetics of the p53 Pathway Affect Ovarian Cancer Incidence and Survival. Clin Cancer Res 2008;14(1):89-96.

(10) Lambeck A, Leffers N, Hoogeboom BN, Sluiter W, Hamming I, Klip H, et al. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007;121(3):606-14.

(11) Abendstein B, Marth C, Muller-Holzner E, Widschwendter M, Daxenbichler G, Zeimet AG. Clinical significance of serum and ascitic p53 autoantibodies in epithelial ovarian carcinoma. Cancer 2000;88(6):1432-7.

(12) Mayerhofer K, Tempfer C, Kucera E, Hefler L, Zeisler H, Kainz C, et al. Humoral p53 antibody response is a prognostic parameter in ovarian cancer. Anticancer Res 1999;19(1B):875-8.

(13) Gadducci A, Ferdeghini M, Buttitta F, Cosio S, Fanucchi A, Annicchiarico C, et al. Assessment of the prognostic relevance of serum anti-p53 antibodies in epithelial ovarian cancer. Gynecol Oncol 1999;72(1):76-81.

(14) Goodell V, Salazar LG, Urban N, Drescher CW, Gray H, Swensen RE, et al. Antibody immunity to the p53 oncogenic protein is a prognostic indicator in ovarian cancer. J Clin Oncol 2006;24(5):762-8.

(15) Vogl FD, Frey M, Kreienberg R, Runnebaum IB. Autoimmunity against p53 predicts invasive cancer with poor survival in patients with an ovarian mass. Br J Cancer 2000;83(10):1338-43.

(16) Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21(2):137-48.

(17) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6(10):715-27.

(18) Atkins D, Breuckmann A, Schmahl GE, Binner P, Ferrone S, Krummenauer F, et al. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer 2004;109(2):265-73.

(19) Atkins D, Ferrone S, Schmahl GE, Storkel S, Seliger B. Down-regulation of HLA class I antigen processing molecules: an immune escape mechanism of renal cell carcinoma? J Urol 2004;171(2 Pt 1):885-9.

(20) Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, Ferrone S. Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. Crit Rev Immunol 2004;24(4):267-96.

(21) Miyagi T, Tatsumi T, Takehara T, Kanto T, Kuzushita N, Sugimoto Y, et al. Impaired expression of proteasome subunits and human leukocyte antigens class I in human colon cancer cells. J Gastroenterol Hepatol 2003;18(1):32-40.

(22) Ritz U, Momburg F, Pilch H, Huber C, Maeurer MJ, Seliger B. Deficient expression of components of the MHC class I antigen processing machinery in human cervical carcinoma. Int J Oncol 2001;19(6):1211-20.

(23) So T, Takenoyama M, Mizukami M, Ichiki Y, Sugaya M, Hanagiri T, et al. Haplotype loss of HLA class I antigen as an escape mechanism from immune attack in lung cancer. Cancer Res 2005;65(13):5945-52.

(24) Vitale M, Pelusi G, Taroni B, Gobbi G, Micheloni C, Rezzani R, et al. HLA class I antigen down-regulation in primary ovary carcinoma lesions: association with disease stage. Clin Cancer Res 2005;11(1):67-72.

(25) Rolland P, Deen S, Scott I, Durrant L, Spendlove I. Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer. Clin Cancer Res 2007;13(12):3591-6.

(26) Cancer Committee of the Internaltional Federation of Gynaecology and Obstetrics. Staging announchement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.

(27) Scully RE. Histological Typing of Ovarian Tumours. 2 ed. Berlin: Springer; 1999.

(28) Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4(7):844-7.

(29) Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol 2004;17(7):790-7.

(30) Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005;102(51):18538-43.

(31) Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res 2006;12(3 Pt 1):878-87.

(32) Berd D, Maguire HC, Jr., Mastrangelo MJ. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res 1986;46(5):2572-7.

(33) Kuball J, Schuler M, Antunes Ferreira E, Herr W, Neumann M, Obenauer-Kutner L, et al. Generating p53-specific cytotoxic T lymphocytes by recombinant adenoviral vector-based vaccination in mice, but not man. Gene Ther 2002;9(13):833-43.

(34) Lomas M, Liauw W, Packham D, Williams K, Kelleher A, Zaunders J, et al. Phase I clinical trial of a human idiotypic p53 vaccine in patients with advanced malignancy. Ann Oncol 2004;15(2):324-9.

(35) Menon AG, Kuppen PJ, van der Burg SH, Offringa R, Bonnet MC, Harinck BI, et al. Safety of intravenous administration of a canarypox virus encoding the human wild-type p53 gene in colorectal cancer patients. Cancer Gene Ther 2003;10(7):509-17.

(36) Nagorsen D, Thiel E. Clinical and immunologic responses to active specific cancer vaccines in human colorectal cancer. Clin Cancer Res 2006;12(10):3064-9.

(37) Svane IM, Pedersen AE, Johansen JS, Johnsen HE, Nielsen D, Kamby C, et al. Vaccination with p53 peptide-pulsed dendritic cells is associated with disease stabilization in patients with p53 expressing advanced breast cancer; monitoring of serum YKL-40 and IL-6 as response biomarkers. Cancer Immunol Immunother 2007;56(9):1485-99.



(38) van der Burg SH, Menon AG, Redeker A, Bonnet MC, Drijfhout JW, Tollenaar RAEM, et al. Induction of p53-specific Immune Responses in Colorectal Cancer Patients Receiving a Recombinant ALVAC-p53 Candidate Vaccine. Clin Cancer Res 2002;8(5):1019-27.

(39) Ehtesham M, Samoto K, Kabos P, Acosta FL, Gutierrez MA, Black KL, et al. Treatment of intracranial glioma with in situ interferon-gamma and tumor necrosis factor-alpha gene transfer. Cancer Gene Ther 2002;9(11):925-34.

(40) Indrova M, Bubenik J, Mikyskova R, Vonka V, Smahel M, Zak R, et al. Tumour-inhibitory and antimetastatic effects of IL-2 in mice carrying MHC class I- tumours of HPV16 origin. Int J Oncol 2002;20(3):643-6.

(41) Fonsatti E, Nicolay HJM, Sigalotti L, Calabro L, Pezzani L, Colizzi F, et al. Functional Up-regulation of Human Leukocyte Antigen Class I Antigens Expression by 5-aza-2'-deoxycytidine in Cutaneous Melanoma: Immunotherapeutic Implications. Clin Cancer Res 2007;13(11):3333-8.

(42) Schrump DS, Fischette MR, Nguyen DM, Zhao M, Li X, Kunst TF, et al. Phase I Study of Decitabine-Mediated Gene Expression in Patients with Cancers Involving the Lungs, Esophagus, or Pleura. Clin Cancer Res 2006;12(19):5777-85.

(43) Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. Proc Natl Acad Sci U S A 2003;100(15):8862-7.

# Chapter 4

## Downregulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer.



Leffers N, Gooden MJM, Mokhova AA,Kast WM, Boezen HM, ten Hoor KA, Hollema H, Daemen T, van der Zee AGJ, Nijman HW.

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## ABSTRACT

### Objective

To investigate the expression and to determine the prognostic impact of components of the antigen processing and presentation pathway (APPP) in ovarian cancer.

### Methods

Expression of MB1, LMP7, TAP1, TAP2, ERp57, ERAP1, β2-microglobulin and the α-chains, HLA-B/C and HLA-A, of the MHC class I molecules was evaluated on tissue microarrays containing primary tumor samples from 232 FIGO stage I-IV ovarian cancer patients. Expression levels were correlated to clinicopathological data and disease-specific (DSS) survival.

### Results

Patients with expression of all components of the MHC class I complex, i.e. HLA-$A^+$ - $\beta_2$-$m^+$ and HLA-$B/C^+$ - $\beta_2$-$m^+$ patients, more often had expression of LMP7, a component of the immunoproteasome than patients with other phenotypes ($p<0.001$). These patients were also more prone to loss of MB1, part of the constitutive multicatalytic proteasome ($P < .05$). Nuclear MB1 expression was an independent predictor of worse DSS (HR 1.94, 95% CI 1.16-3.26, p=0.012). The HLA-$B/C^+$ - $\beta_2$-$m^+$ phenotype was an independent predictor of a better prognosis (HR 0.63, 95% CI 0.40-0.99, p=0.047). Median DSS was longer for patients with normal nuclear expression of LMP7 (57.4 vs. 31.0 months, p=0.029).

### Conclusions

The prognostic influence of the proteasomal subunit MB1 and the MHC class I complex in ovarian cancer provide a rationale for targeting these specific APPP components in ovarian cancer.

## INTRODUCTION

Ovarian cancer is the second most common gynecological cancer and the leading cause of death from gynecological malignancies in the Western world (1). The exploration of immunotherapy as a new treatment modality for this disease relies on evidence of improved clinical outcome when intra-tumoral T-lymphocytes are present in ovarian cancer patients, which is most likely a reflection of an anti-tumor immune response (2). Immunotherapy can potentially overcome the poor survival rate seen with standard treatment, i.e. surgical debulking and adjuvant platinum based chemotherapy (3;4), by inducing or augmenting anti-tumor immune responses. Different strategies have been employed, including adoptive transfer of anti-tumor T cells or natural killer (NK) cells, as well as active immunization with tumor antigens, such as p53. Contrary to some promising murine studies (5-7), immunotherapeutic trials in human subjects have only reached minimal clinical benefit (8;9). This may partly be due to the development and exploitation of immune escape mechanism by the tumor. Down-regulation or absence of components of the antigen processing and presentation pathway (APPP) is believed to be such mechanism as presentation of antigens either processed within the MHC class I or the MHC class II pathway is a prerequisite for recognition by $CD8^+$ cytotoxic and $CD4^+$ helper T-lymphocytes, respectively (10;11).



The mainstay of the anti-tumor immune response is via MHC class I. In the MHC class I pathway, cytoplasmic and nuclear proteins are degraded by the interferon-γ inducible subunits LMP7, LMP2 and LMP10 (components of the immunoproteasome), which can replace subunits MB1(X), delta(Y) and zeta(Z) of the multicatalytic constitutive proteasome (10-14). Immunoproteasomes are generally thought to be more efficient at the production of antigenic peptides than the constitutive proteasome (13) and they tend to produce more and on average slightly longer peptides (15). Immunoproteasomes are preferentially located near the endoplasmic reticulum (ER), whereas the constitutive proteasome can be found throughout the cytoplasm (16). Proteasomes may also be present in the nucleus, especially in case of cell stress (17). Next peptides of a suitable length are transported from the cytoplasm into the endoplasmic reticulum by the transporter associated with antigen processing (TAP), consisting of subunits TAP1 and TAP2 (10;11;13;14;18). Once in the ER, aminopeptidases such as endoplasmic reticulum aminopeptidase 1 (ERAP1) can further trim peptides to a correct size for adequate MHC class I binding. Chaperones (e.g. ERp57) facilitate loading of peptides into the MHC class I molecule (10;11;19-21). Finally, a fully assembled heterotrimeric MHC class I/peptide complex (α-chain, β2-microglobulin and a bound antigenic peptide) is transported to the cell surface, where it may be recognized by CD8+ cytotoxic T lymphocytes (11;13;14;20). A schematic overview of this pathway is given in figure 1.

Although defects and down-regulation in all of the aforementioned components have been identified in several types of cancer, most APPP defects have some, little or no significant relation with clinical parameters and disease outcome (11;22-32). In ovarian cancer, a study of five APPP components recently showed that the number of down-regulated components is an independent prognostic factor (33). We investigated the expression and clinical relevance of these and essential additional components of the APPP in paraffin embedded tissues obtained from a large series of well-documented ovarian cancer patients.



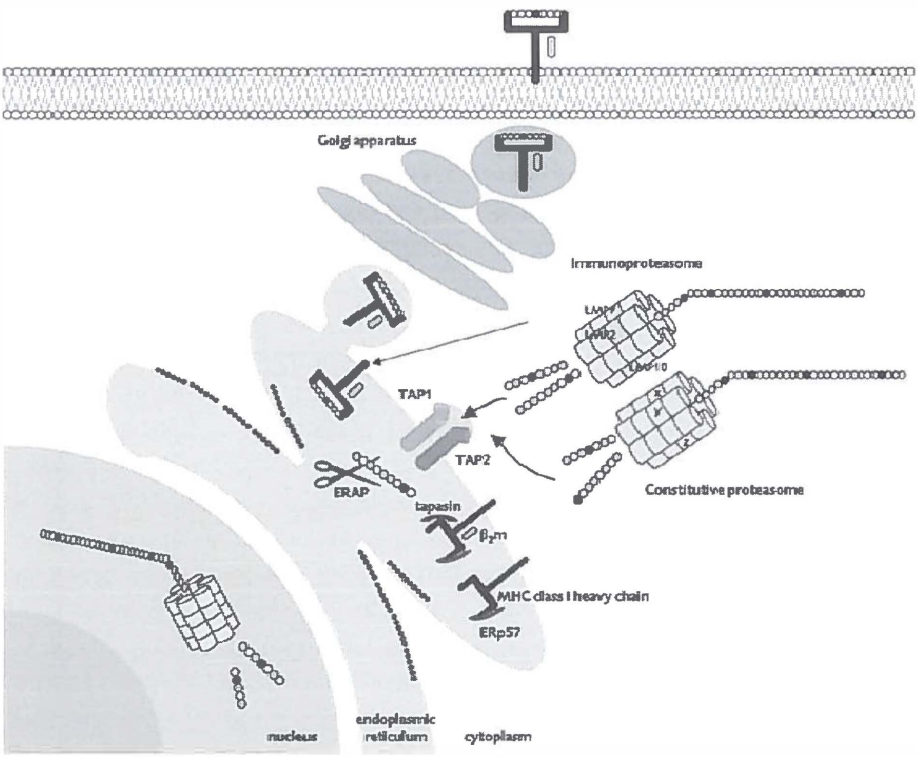



**Fig. 1** Schematic overview of MCH class I dependent antigen processing and presentation pathway.

## MATERIALS AND METHODS

### Patients

Patients were identified from a database containing clinicopathological and follow-up data of all epithelial ovarian cancer treated with primary debulking surgery according to standard treatment protocols by gynecological oncologists of the University Medical Center Groningen (Groningen, The Netherlands) between May 1985 and April 2003. Patients were selected if sufficient paraffin embedded tissue was available. All patients were staged according to the FIGO classification, and resected tumors were graded and classified by a gynecological pathologist based on the World Health Organization criteria. Adjuvant chemotherapy was given if indicated. Follow-up of all patients was performed regularly up to ten years with gradually increasing intervals.



### Institutional review board approval

All clinicopathologic and follow-up data of patients referred to the Department of Gynecologic Oncology of the UMCG are prospectively collected and stored in a computerized database. For the present study, all relevant data were retrieved from our computerized database and transferred into a separate password-protected anonymous database. Patient identity was protected by study-specific unique patient codes. Patients' true identity was only known to two dedicated data managers, who have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the data managers. Based on this information, according to Dutch law no further approval from our Institutional Review Board was needed.

### Tissue samples and construction of tissue microarrays

Paraffin-embedded tissue blocks and corresponding hematoxylin & eosin stained slides constructed from tumor tissue obtained at primary debulking surgery were retrieved from the pathology archives. Tissue microarrays (TMA) were constructed as described previously (34-36). Briefly, primary tumor samples were arrayed using a tissue microarrayer (Beecher Instruments, Silver Spring, Maryland) by taking four representative cores with a diameter of 0.6 mm from marked tumor sites from the individual paraffin embedded tumor block onto a recipient paraffin block at pre-defined array locations. For staining, 4µm sections were cut from each TMA block and applied to APES-coated slides. H&E staining was performed to verify the presence of tumor in the arrayed samples.

**Table 1** Clinicopathological characteristics and survival data

| | Total (n=232) | Early stage (n=64) | Late stage (n=166) |
|---|---|---|---|
| *Age (years)* | | | |
| Mean (SD) | 57.7 (14.2) | 53.1 (14.5) | 59.7 (13.6) |
| *5 year DSS* | 39% | 79% | 25% |
| *FIGO Stage* | | | |
| Stage I | 45 (19.4%) | 45 (70.3%) | |
| Stage II | 19 (8.2%) | 19 (29.7%) | |
| Stage III | 133 (57.3%) | | 133 (80.1%) |
| Stage IV | 33 (14.2%) | | 33 (19.9%) |
| Missing | 2 (0.9%) | | |
| *Tumour type* | | | |
| Serous | 128 (55.2%) | 13 (20.3%) | 115 (69.3%) |
| Mucinous | 27 (11.6%) | 18 (28.1%) | 8 (4.8%) |
| Endometrioid | 33 (14.2%) | 19 (29.7%) | 14 (8.4%) |
| Clear Cell | 17 (7.3%) | 6 (9.4%) | 10 (6.0%) |
| Adenocarcinoma | 9 (3.9%) | 2 (3.1%) | 7 (4.2%) |
| Mixed Tumours | 10 (4.3%) | 5 (7.8%) | 5 (3.0%) |
| Other | 8 (3.4%) | 1 (1.6%) | 7 (4.2%) |
| *Tumour Grade* | | | |
| Grade I | 39 (16.8%) | 29 (45.3%) | 9 (5.4%) |
| Grade II | 52 (22.4%) | 22 (34.4%) | 29 (17.5%) |
| Grade III | 104 (44.8%) | 7 (10.9%) | 97 (58.4%) |
| Undifferentiated | 14 (6.0%) | 2 (3.1%) | 12 (7.2%) |
| Missing | 23 (9.9%) | 4 (6.3%) | 19 (11.4%) |
| *Residual disease* | | | |
| < 2 cm | 119 (51.3%) | 62 (96.9%) | 55 (33.1%) |
| >= 2 cm | 109 (47.0%) | 2 (3.1%) | 107 (64.5%) |
| Missing | 4 (1.7%) | 0 (0.0%) | 4 (2.4%) |
| *Type of chemotherapy* | | | |
| No chemotherapy | 32 (13.8%) | 21 (32.8%) | 11 (6.6%) |
| Platinum containing | 102 (44.0%) | 22 (34.4%) | 79 (47.6%) |
| Platinum & taxane containing | 72 (31.0%) | 13 (20.3%) | 59 (35.5%) |
| Other | 24 (10.3%) | 7 (10.9%) | 17 (10.2%) |
| Missing | 2 (0.9%) | 1 (1.6%) | |

DSS = disease-specific survival; FIGO = International Federation of Gynaecology and Obstetrics.

**Immunohistochemical staining**

TMA sections were dewaxed and rehydrated, whereupon antigen retrieval was performed in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by a 3% $H_2O_2$ solution, after which sections were incubated overnight at 4 ºC with the primary antibodies in the following dilutions: anti-MB1 (clone SJJ-3) 1:10, anti-LMP7 1: 100 (clone 1B3, Novus Biologicals, Heerhugowaard, The Netherlands), anti-TAP1 1:20 (clone H-300, Santa Cruz Biothechnology Inc., Heidelberg, Germany), anti-TAP2 1:20 (clone H-210, Santa Cruz Biothechnology Inc., Heidelberg, Germany), anti-ERp57 (clone TO-2) 1: 20, polyclonal anti-ERAP1 1:100 , polyclonal anti-$\beta_2$-microglobulin 1:400 ( DAKO, Glostrup, Denmark) and anti-MHC class I heavy chain antibodies HCA2 1: 500 (HLA-A) and HC-10 1:100 (HLA-B/C). The anti-ERp57 and anti-MB1 antibodies were a gift from Dr S. Ferrone, University of Pittsburgh, Pittsburgh, PA. The HCA2 and HC-10 antibodies were a gift from Dr. J.J. Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands. The anti-ERAP1 antibody was a gift from Dr. M. Tsujimoto, Riken, Wako, Saitama, Japan. Sections were subsequently incubated with DAKO Envision+ (Dako, Heverlee, Belgium). Antigen-antibody reactions were visualized with 3,3-diaminobenzidine, the chromogenic substrate for peroxidase, and hematoxylin was used to counterstain the tissue.

**Evaluation of immunostaining**

Scoring was done independently by two investigators, without prior knowledge of the clinicopathological parameters. The semi-quantitative quality control system proposed by Ruiter et al. (37) was used, in which both intensity of staining and percentage of positive tumor cells are determined. The intensity of staining was scored as 0, 1, 2, or 3, indicating absent, weak, positive, or strong positive expression, respectively. The percentage of positive cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The sum of both scores was used to identify three categories of expression: normal expression (total score 7–8), partial loss (3–6) and total loss (0–2), as previously described by Mehta et al. (28). Immunohistochemical staining demonstrated strong positive expression of all examined markers in stromal tissue and tumor-infiltrating inflammatory cells, thereby providing an internal positive control.

As the proteasome containing the MB1 or LMP7 subunit may be present in both the nucleus and cytoplasm (17) and a difference in intensity was apparent between these organelles, both were scored separately. Staining of ERp57, ERAP1, TAP1, and TAP2 was evaluated in the cytoplasm, while for $\beta_2$-microglobulin, MHC class I α (heavy) chain (HC-10 and HC-A2) cell membrane staining was graded.

**Statistical Analysis**

Patients were entered into analyses only when at least two cores with viable tumor cells were available. To perform statistical analyses, expression levels were dichotomized. Thus, for MB1, LMP7, ERp57, and ERAP1, patients with total and partial loss were taken together. TAP1 and TAP2 expression were naturally dichotomous, since no total loss was observed. Therefore, partial loss was set off against normal expression. Finally, components of MHC class I complex were dichotomized by comparing patients with a heavy chain$^+$ - $\beta_2$-m$^+$ phenotype to patients with all other phenotypes. Associations between expression levels of the APPP components and clinicopathological parameters were tested using the $\chi^2$ test. Differences in disease-specific survival (DSS) based on expression levels were plotted using Kaplan-Meier survival curves and evaluated by log-rank tests. DSS was defined as the time from primary debulking surgery until death due to ovarian cancer or the date of last follow-up. Subsequently, multivariate analysis was performed with Cox proportion hazards model and was stratified for type of chemotherapy. Only those variables that were significantly associated with DSS in univariate analyses, were entered into the Cox proportion hazards model.

All analyses were performed using SPSS version 14.0 software package for windows (SPSS Inc., Chicago, USA). P values <0.05 were considered significant (tested 2-sided).

**Table 2** Expression levels of APM components

| | Normal | Partial loss | Total loss | Missing |
|---|---|---|---|---|
| *MB1 nucleus* | 156 (67.2%) | 55 (23.7%) | 10 (4.3%) | 11 (4.7%) |
| *MB1 cytoplasm* | 127 (54.7%) | 88 (37.9%) | 6 (2.6%) | 11 (4.7%) |
| *LMP7 nucleus* | 88 (37.9%) | 100 (43.1%) | 34 (14.7%) | 10 (4.3%) |
| *LMP7 cytoplasm* | 56 (24.1%) | 131 (56.5%) | 35 (15.1%) | 10 (4.3%) |
| *TAP1* | 189 (81.5%) | 24 (10.3%) | 0 (0%) | 19 (8.2%) |
| *TAP2* | 185 (79.7%) | 28 (12.1%) | 0 (0%) | 19 (8.2%) |
| *ERp57* | 147 (63.4%) | 74 (31.9%) | 3 (1.3%) | 8 (3.4%) |
| *ERAP1* | 183 (78.9%) | 39 (16.8%) | 0 (0%) | 10 (4.3%) |
| | | **Both components** | **All other phenotypes** | **Missing** |
| *Co-expression of HLA-A and $\beta_2$-m* | | 114 (49.1%) | 102 (44.0%) | 16 (6.9%) |
| *Co-expression of HLA-B/C and $\beta_2$-m* | | 162 (69.8%) | 56 (24.1%) | 14 (6.0%) |

## RESULTS

### Patient characteristics

Paraffin embedded tumor tissue was available for 259 ovarian cancer patients. Twenty-seven patients were excluded because no samples were available from primary debulking surgery. Patient characteristics are summarized in table 1. The majority of patients presented with serous histology, late stage, and/or high grade disease. Thirty-two patients did not receive chemotherapy, 21 of whom were diagnosed with early stage disease. The remaining 11 patients were either unfit or unwilling to receive chemotherapy. Of the patients treated with chemotherapy 87.9% received a platinum-based regimen. Estimated five-year DSS rate was 39%. Median DSS was 33.8 months (95% C.I.: 22.2-45.4).

### Expression levels of APPP components

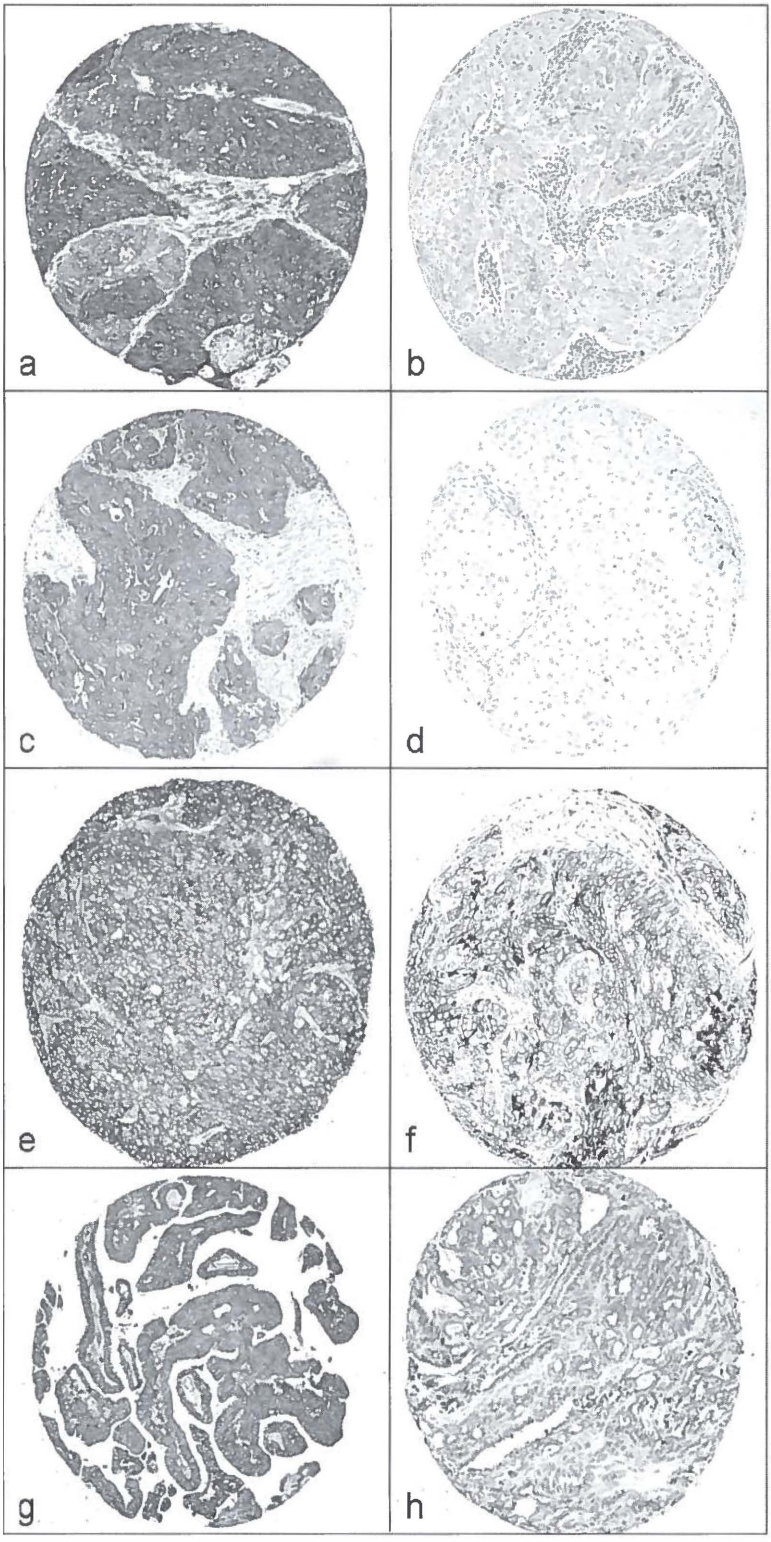
Expression levels of all APPP components are shown in table 2. Representative staining patterns of several components are depicted in figure 2. Percentages of missing data vary from 3.4-8.2%. Partial and/or total loss of expression was witnessed for all evaluated components.

The presence of a heavy chain and $\beta_2$-m are prerequisites for the formation of a stable MHC class I complex; co-expression of HLA-A and $\beta_2$-m and HLA-B/C and $\beta_2$-m was observed in 49.1%, respectively 69.8% of patients. Relationships between HLA-A - $\beta_2$-m or HLA-B/C - $\beta_2$-m and other APPP components are shown in table 3. For both combinations, there was a striking paradox between MB1 and LMP7 expression, i.e. the majority of HLA-$A^+$ - $\beta_2$-$m^+$ and HLA-$B/C^+$ - $\beta_2$-$m^+$ patients had normal expression of nuclear and cytoplasmic LMP7, a component of the immunoproteasome, whereas these patients were more often prone to loss of nuclear and/or cytoplasmic MB1, part of the constitutive multicatalytic proteasome. Surprisingly, no relationship existed between MB1 and LMP7 expression (data not shown).

We found that HLA-$A^+$ - $\beta_2$-$m^+$ patients were also more likely to show normal expression of TAP1, ERp57, and ERAP1 than patients with loss of MHC class I components. HLA-$B/C^+$ - $\beta_2$-$m^+$ patients often expressed TAP1 and ERAP1 while these components tended to be down-regulated in patients who lacked one or both components of the MHC class I complex (table 3).

### Association with clinicopathological factors

Loss of nuclear LMP7 was more frequently observed in patients ≥58 years old (67.8 vs. 51.5%, p=0.014; data not shown), as was the case for loss of cytoplasmic LMP7 (82.6 vs. 65.3%, p=0.003). The HLA-$A^+$ - $\beta_2$-$m^+$ phenotype was more frequently observed in patients with FIGO stage III/IV disease (70.5 vs. 55.9%, p=0.043; data not shown).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**Association with disease-specific survival**

Median DSS was 51.5 months shorter for patients with normal nuclear expression of MB1 as opposed to patients with loss of nuclear MB1 (figure 3a; median DSS 27.0 vs. 78.5 months, p=0.005). Likewise, DSS was shorter for patients with normal cytoplasmic expression of MB1 (figure 3b; median DSS 30.5 vs. 60.2 months, p=0.024). On the contrary, nuclear expression of LMP7 was associated with improved DSS (figure 3c; median DSS 57.4 vs. 31.0 months, p=0.029).

DSS was more than twice as long in patients with the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype as opposed to patients with any another phenotype (figure 3d; median 45.2 vs. 21.1 months, p=0.015).

We repeated the univariate survival analyses in a subgroup containing 166 patients with advanced stage disease only (data not shown). Improved DSS was demonstrated based on down-regulation of nuclear MB1 expression (median 45.2 vs. 17.8 months, p=0.008) and the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype (median 25.7 vs. 13.4, p=0.028).



**Multivariate analysis**

Only the above-mentioned variables that were significantly associated with DSS in the univariate analyses were entered into the Cox proportion hazards model, which was additionally adjusted for well-known prognostic factors (table 4).

After stratification for type of chemotherapy, normal nuclear MB1 expression was found to be an independent prognostic factor for shortened DSS (HR 1.94, 95% CI 1.16-3.26, p=0.012). Furthermore, the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype was demonstrated to be an independent predictor of longer DSS (HR 0.63, 95% CI 0.40-0.99, p=0.047). Likewise, MB1 expression and the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype were found to be independent predictors of DSS in the advanced stage subgroup (HR 1.78, 95% CI 1.06-2.98, p=0.028, resp. HR 0.55, 95% CI 0.35-0.87, p=0.010).

**Fig. 2** Representative example of positive and negative/weakly positive staining pattern of several APPP components. a, b) MB1; c, d) LPM7; e, f) HLA-B/C; and g, h) β2-m expression by ovarian tumor cells (400x).

**Table 3** Associations of MHC class I components with other APPP components

| | Co-expression HLA-A and $\beta_2$-m | | | Co-expression HLA-B/C and $\beta_2$-m | | |
|---|---|---|---|---|---|---|
| | HLA-A$^+$/β2-m$^+$ | all other phenotypes | p-value[a] | HLA-B/C$^+$/β2-m$^+$ | all other phenotypes | p-value[a] |
| *N* = | 114 | 102 | | 162 | 56 | |
| *MB1 nucleus* | | | | | | |
| normal expression | 73 (48.7%) | 77 (51.3%) | **0.048** | 102 (67.5%) | 49 (32.5%) | **<0.001** |
| loss of expression | 40 (63.5%) | 23 (36.5%) | | 58 (90.6%) | 6 (9.4%) | |
| *MB1 cytoplasm* | | | | | | |
| normal expression | 56 (45.9%) | 66 (54.1%) | **0.015** | 81 (66.4%) | 41 (33.6%) | **0.002** |
| loss of expression | 57 (62.6%) | 34 (37.4%) | | 79 (84.9%) | 14 (15.1%) | |
| *LMP7 nucleus* | | | | | | |
| normal expression | 65 (76.5%) | 20 (23.5%) | **<0.001** | 78 (91.8%) | 7 (8.2%) | **<0.001** |
| loss of expression | 48 (36.9%) | 82 (63.1%) | | 83 (62.9%) | 49 (37.1%) | |
| *LMP7 cytoplasm* | | | | | | |
| normal expression | 39 (72.2%) | 15 (27.8%) | **0.001** | 48 (88.9%) | 6 (11.1%) | **0.004** |
| loss of expression | 74 (46.0%) | 87 (54.0%) | | 113 (69.3%) | 50 (30.7%) | |
| *TAP1* | | | | | | |
| normal expression | 109 (59.9%) | 73 (40.1%) | **<0.001** | 143 (77.7%) | 41 (22.3%) | **0.001** |
| loss of expression | 2 (8.3%) | 22 (91.7%) | | 11 (45.8%) | 13 (54.2%) | |
| *TAP2* | | | | | | |
| normal expression | 97 (56.1%) | 76 (43.9%) | 0.065 | 133 (76.0%) | 42(24.0%) | 0.299 |
| loss of expression | 10 (37.0%) | 17 (63.0%) | | 18 (66.7%) | 9 (33.3%) | |
| *ERp57* | | | | | | |
| normal expression | 88 (62.0%) | 54 (38.0%) | **<0.001** | 110 (76.4%) | 34 (23.6%) | 0.328 |
| loss of expression | 26 (35.1%) | 48 (64.9%) | | 52 (70.3%) | 22 (29.7%) | |
| *ERAP1* | | | | | | |
| normal expression | 107 (60.1%) | 71 (39.9%) | **<0.001** | 139 (77.7%) | 40 (22.3%) | **0.016** |
| loss of expression | 7 (18.4%) | 31 (81.6%) | | 23 (59.0%) | 16 (41.0%) | |

For each comparison, patients were entered into analysis only when information was available on the heavy chain - β2-m phenotype as well as on a specific APPP component. [a] Associations between APPP components and fully assembled MHC class I complexes were evaluated using the Chi square-test. Bold signifies $p<0.05$.

**Table 4** Multivariate Cox regression analysis of disease-specific survival in ovarian cancer patients*

| | MB1 expression | | | MB1 expression | | | LMP7 expression | | | HLA-B/C - $\beta_2$-m phenotype | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HR | 95% CI | p-value | HR | 95% CI | p-value | HR | 95% CI | p-value | HR | 95% CI | p-value |
| Age ≥ 58 years | 1.19 | 0.76-1.87 | 0.440 | 1.16 | 0.74-1.82 | 0.510 | 1.04 | 0.67-1.63 | 0.859 | 1.05 | 0.40-0.99 | 0.047 |
| Grade III / undiff | 2.09 | 1.26-3.47 | 0.055 | 1.94 | 1.16-3.22 | **0.011** | 1.72 | 1.04-2.82 | **0.033** | 1.92 | 1.16-3.20 | **0.012** |
| Non-serous tumor | 0.48 | 0.28-0.82 | **0.008** | 0.50 | 0.29-0.87 | **0.014** | 0.51 | 0.30-0.86 | **0.012** | 0.52 | 0.30-0.89 | **0.016** |
| FIGO stage III/IV | 2.99 | 1.15-7.74 | **0.024** | 3.11 | 1.19-8.15 | **0.021** | 2.96 | 1.18-7.39 | **0.020** | 2.93 | 1.17-7.34 | **0.022** |
| Residual tumor | 2.45 | 1.53-3.93 | **<0.001** | 2.46 | 1.54-3.93 | **<0.001** | 2.60 | 1.61-4.20 | **<0.001** | 2.48 | 1.55-3.98 | **<0.001** |
| MB1$^+$ (nucleus) | 1.94 | 1.16-3.26 | **0.012** | | | | | | | | | |
| MB1$^+$ (cytoplasm) | | | | 1.40 | 0.89-2.22 | 0.150 | | | | | | |
| LMP7$^+$ (nucleus) | | | | | | | 0.73 | 0.47-1.13 | 0.163 | | | |
| HLA-B/C$^+$ / $\beta_2$-m$^+$ | | | | | | | | | | 0.63 | 0.40-0.99 | **0.047** |

*Results represent four separate multivariate analyses including five well-known prognostic factors and one APPP component at a time. All analyses were performed stratified for type of chemotherapy; FIGO = International Federation of Gynecology and Obstetrics; HR = hazard ratio; CI = confidence interval; bold signifies p<0.05.

## DISCUSSION

The antigen processing and presentation pathway bridges many cellular organelles, ultimately resulting in the MHC restricted presentation of a small peptide sequence on the cell surface. Recognition of this peptide-MHC complex by the immune system can lead to a potentially life-prolonging anti-tumour immune response (2). Individual components of the APPP may have a prognostic value and/or a therapeutic potential. At the level of the proteasome, our study indicates that the presence of MB1 is an independent predictor of shorter DSS in ovarian cancer. In contrast, expression of LMP7 is associated with longer DSS. At the cell membrane level, the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype is a predictor of longer DSS. Furthermore, patients with heavy chain and $\beta_2$-m expression are more likely to express LMP7, while patients lacking one of the MHC class I components more often express MB1.



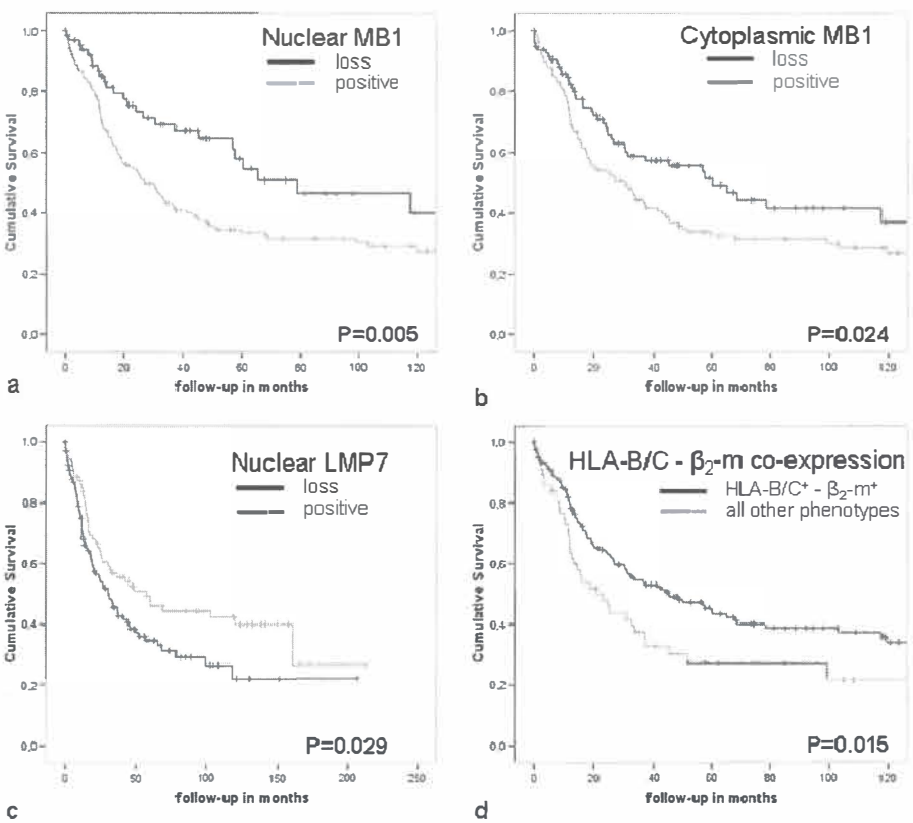


**Fig. 3** Disease-specific survival (in months) of FIGO stages I–IV ovarian cancer.
Cumulative survival time was estimated by the Kaplan-Meier method. Log Rank test was used to evaluate survival differences between groups. (A) Nuclear and (B) cytoplasmic expression of MB1 is associated with worse disease-specific survival. (C) Nuclear expression of LMP7 is associated with better disease-specific survival. (D) Better disease-specific survival was observed in patients with the HLA-B/C–β2-m+ phenotype.

Whereas previous studies reported mainly on the immunoproteasome (22;27;28;38;39), we observed an important role for the constitutive proteasome. To our knowledge, the negative influence of MB1 expression on prognosis of (ovarian) cancer patients has not previously been described, and opposes the results of a study in renal cell carcinoma, where MB1 expression did not influence survival (39). It has been suggested that there is a reciprocal relationship between MB1 and LMP7, which may explain the observed negative prognostic influence of MB1 expression (39;40). We were, however, unable to find such an association between expression levels of LMP7 and MB1.

An alternative explanation for the survival benefit associated with MB1 loss might be a mechanism similar to that observed with proteasome inhibitors, e.g. Bortezomib (41). These agents inhibit the ubiquitin-proteasome pathway in tumour cells, resulting in accumulation of proteins involved in cell cycle regulation, proliferation, differentiation, and apoptosis. Ultimately, proteasome inhibitors increase apoptosis and decrease chemoresistance (42). Patients in whom the constitutive proteasome is intrinsically down-regulated might enjoy a similar benefit.

Nuclear expression of the immunoproteasome component LMP7 was associated with longer DSS. Similarly, differences in DSS based on LMP7 expression have been reported for several types of cancer (28;38;39). These survival benefits can be interpreted as the result of immunologic mechanisms by which the generation of more immunogenic peptides results in more efficient activation of the immune system. Since LMP7 expression conveys a positive effect on DSS, proteasome inhibition might not be beneficial for patients expressing LMP7, which suggests that more selective proteasome inhibitors may be needed for optimal clinical benefit. Alternatively, one could envision treating patients with agents that enhance LMP7 and/or MHC class I expression. Histone deacetylase inhibitors, e.g. trichostatin A and valproic acid, have been shown to have these properties, but more specific compounds would be desirable (43).

The final step of the antigen processing and presentation pathway is cell surface expression of a fully assembled heterotrimeric MHC class I complex consisting of a heavy chain molecule, $\beta_2$-microglobulin, and a bound antigenic peptide. In this study, expression of the HLA-A and HLA-B/C heavy chain molecules was analyzed in combination with $\beta_2$-microglobulin. We observed that the HLA-B/C$^+$ - $\beta_2$-m$^+$ phenotype is an independent positive prognostic factor for DSS, confirming the recently published findings of Rolland and colleagues in ovarian cancer (44).

Strikingly, we found that MB1 expression correlates with loss of the heavy chain$^+$/ $\beta_2$-m$^+$ phenotype, while patients with LMP7 are likely to express the fully assembled MHC class I complex. This implies that in cases where the immunoproteasome dominates over the constitutive proteasome, effective antigen presentation is more likely. Moreover, the presence of upstream APPP components could be necessary

for formation of a stable MHC class I complex (45). Thus, the generation of suboptimal peptide sequences by the constitutive proteasome is exacerbated by impaired presentation of the peptide on the cell surface, resulting in escape from immune detection, thereby promoting 'immunoediting', i.e. the selective outgrowth of tumour components with low immunogenicity. To further substantiate this, the association of expression of the (immuno)proteasome to tumor-infiltrating lymphocytes was determined using the results from a recently published study of tumour-infiltrating lymphocytes in the same population (46). Higher numbers of tumour-infiltrating $CD8^+$ T-lymphocytes are more frequently observed in patients with loss of MB1 expression as well as in patients with positive LMP7 expression (data not shown). Whether down-regulation of the constitutive and up-regulation of the immunoproteasome result in an increase in lymphocyte infiltration, or whether the presence of interferon-γ producing lymphocytes facilitates the switch from constitutive to immunoproteasome needs to be elucidated.



While we found prognostic significance associated with components at either the beginning or the very end of the APPP, we did not detect any survival differences based on expression levels of proteins active at the level of the endoplasmatic reticulum. Summation of APPP components, however, similar to the approach of Han et al. (33) indeed correlated with worse survival in patients with down-regulation of one or more components as opposed to $TAP1^+$ $TAP2^+$ HLA-B/$C^+$ $\beta_2$-$microglobulin^+$ patients (data not shown). Furthermore, combination of these results with a tumour-infiltrating lymphocytes revealed that patients with expression of all four components more frequently have an abundance of tumour-infiltrating $CD8^+$ T-lymphocytes. Moreover, we also find that both the absolute number of APPP components and $CD8^+$ T-lymphocytes are independent prognostic predictors of longer disease specific survival, next to some well-acknowledged prognostic factors (33;46).

In conclusion, our results underscore a pivotal role for the (immuno)proteasome and MHC class I complex in determining prognosis in ovarian cancer patients. In view of the complexity of the APPP, these results provide a rationale for targeting these specific components in targeted therapy in cancer.

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## REFERENCES


(1) Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005 Mar;55(2):74-108.

(2) Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003 Jan 16;348(3):203-13.

(3) Coukos G, Conejo-Garcia JR, Roden RB, Wu TC. Immunotherapy for gynaecological malignancies. Expert Opin Biol Ther 2005 Sep;5(9):1193-210.

(4) Guppy AE, Nathan PD, Rustin GJ. Epithelial ovarian cancer: a review of current management. Clin Oncol (R Coll Radiol ) 2005 Sep;17(6):399-411.

(5) Chiriva-Internati M, Weidanz JA, Raffaele F, Yu Y, Frezza EE, Jenkins MR, et al. Adoptive T-cell based immunotherapy eradicates metastatic ovarian cancer. J Immunother. In press 2008.

(6) Mayordomo JI, Loftus DJ, Sakamoto H, De Cesare CM, Appasamy PM, Lotze MT, et al. Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J Exp Med 1996 Apr 1;183(4):1357-65.

(7) Nikitina EY, Chada S, Muro-Cacho C, Fang B, Zhang R, Roth JA, et al. An effective immunization and cancer treatment with activated dendritic cells transduced with full-length wild-type p53. Gene Ther 2002 Mar;9(5):345-52.

(8) Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res 2006 Feb 1;12(3 Pt 1):878-87.

(9) Svane IM, Pedersen AE, Johansen JS, Johnsen HE, Nielsen D, Kamby C, et al. Vaccination with p53 peptide-pulsed dendritic cells is associated with disease stabilization in patients with p53 expressing advanced breast cancer; monitoring of serum YKL-40 and IL-6 as response biomarkers. Cancer Immunol Immunother 2007 Sep;56(9):1485-99.

(10) Bangia N, Ferrone S. Antigen presentation machinery (APM) modulation and soluble HLA molecules in the tumor microenvironment: do they provide tumor cells with escape mechanisms from recognition by cytotoxic T lymphocytes? Immunol Invest 2006;35(3-4):485-503.

(11) Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today 2000 Sep;21(9):455-64.

(12) Coux O, Tanaka K, Goldberg AL. Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem 1996;65:801-47.

(13) Kloetzel PM, Ossendorp F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. Curr Opin Immunol 2004 Feb;16(1):76-81.

(14) Purcell AW, Elliott T. Molecular machinations of the MHC-I peptide loading complex. Curr Opin Immunol 2008 Feb;20(1):75-81.

(15) Cascio P, Hilton C, Kisselev AF, Rock KL, Goldberg AL. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. EMBO J 2001 May 15;20(10):2357-66.

(16) Rivett AJ, Hearn AR. Proteasome function in antigen presentation: immunoproteasome complexes, Peptide production, and interactions with viral proteins. Curr Protein Pept Sci 2004 Jun;5(3):153-61.

(17) Dahlmann B. Role of proteasomes in disease. BMC Biochem 2007;8 Suppl 1:S3.

(18) Ritz U, Momburg F, Pircher HP, Strand D, Huber C, Seliger B. Identification of sequences in the human peptide transporter subunit TAP1 required for transporter associated with antigen processing (TAP) function. Int Immunol 2001 Jan;13(1):31-41.

(19) Chang SC, Momburg F, Bhutani N, Goldberg AL. The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. Proc Natl Acad Sci U S A 2005 Nov 22;102(47):17107-12.

(20) Cresswell P, Androlewicz MJ, Ortmann B. Assembly and transport of class I MHC-peptide complexes. Ciba Found Symp 1994;187:150-62.

(21) Garbi N, Tanaka S, van den Broek M, Momburg F, Hammerling GJ. Accessory molecules in the assembly of major histocompatibility complex class I/peptide complexes: how essential are they for CD8(+) T-cell immune responses? Immunol Rev 2005 Oct;207:77-88.

(22) Atkins D, Breuckmann A, Schmahl GE, Binner P, Ferrone S, Krummenauer F, et al. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer 2004 Mar 20;109(2):265-73.

(23) Atkins D, Ferrone S, Schmahl GE, Storkel S, Seliger B. Down-regulation of HLA class I antigen processing molecules: an immune escape mechanism of renal cell carcinoma? J Urol 2004 Feb;171(2 Pt 1):885-9.



(24) Campoli M, Chang CC, Oldford SA, Edgecombe AD, Drover S, Ferrone S. HLA antigen changes in malignant tumors of mammary epithelial origin: molecular mechanisms and clinical implications. Breast Dis 2004;20:105-25.

(25) Gobbi G, Mirandola P, Micheloni C, Solenghi E, Sponzilli I, Artico M, et al. Expression of HLA class I antigen and proteasome subunits LMP-2 and LMP-10 in primary vs. metastatic breast carcinoma lesions. Int J Oncol 2004 Dec;25(6):1625-9.

(26) Johnsen AK, France J, Nagy N, Askew D, Abdul-Karim FW, Gerson SL, et al. Systemic deficits in transporter for antigen presentation (TAP)-1 or proteasome subunit LMP2 have little or no effect on tumor incidence. Int J Cancer 2001 Feb 1;91(3):366-72.

(27) Kageshita T, Hirai S, Ono T, Hicklin DJ, Ferrone S. Down-regulation of HLA class I antigen-processing molecules in malignant melanoma: association with disease progression. Am J Pathol 1999 Mar;154(3):745-54.

(28) Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren GJ. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. Cancer Immunol Immunother 2008;57:197-206.

(29) Miyagi T, Tatsumi T, Takehara T, Kanto T, Kuzushita N, Sugimoto Y, et al. Impaired expression of proteasome subunits and human leukocyte antigens class I in human colon cancer cells. J Gastroenterol Hepatol 2003 Jan;18(1):32-40.

(30) Ritz U, Momburg F, Pilch H, Huber C, Maeurer MJ, Seliger B. Deficient expression of components of the MHC class I antigen processing machinery in human cervical carcinoma. Int J Oncol 2001 Dec;19(6):1211-20.

(31) Seliger B, Ritz U, Abele R, Bock M, Tampe R, Sutter G, et al. Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. Cancer Res 2001 Dec 15;61(24):8647-50.

(32) So T, Takenoyama M, Mizukami M, Ichiki Y, Sugaya M, Hanagiri T, et al. Haplotype loss of HLA class I antigen as an escape mechanism from immune attack in lung cancer. Cancer Res 2005 Jul 1;65(13):5945-52.

(33) Han LY, Fletcher MS, Urbauer DL, Mueller P, Landen CN, Kamat AA, et al. HLA class I antigen processing machinery component expression and intratumoral T-Cell infiltrate as independent prognostic markers in ovarian carcinoma. Clin Cancer Res 2008 Jun 1;14(11):3372-9.

(34) Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000 Dec;80(12):1943-9.

(35) Hendriks Y, Franken P, Dierssen JW, De LW, Wijnen J, Dreef E, et al. Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors. Am J Pathol 2003 Feb;162(2):469-77.

(36) Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol 2004 Jul;17(7):790-7.

(37) Ruiter DJ, Ferrier CM, Van Muijen GN, Henzen-Logmans SC, Kennedy S, Kramer MD, et al. Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. European BIOMED-1 Concerted Action on Clinical Relevance of Proteases in Tumour Invasion and Metastasis. Eur J Cancer 1998 Aug;34(9):1334-40.

(38) Meissner M, Reichert TE, Kunkel M, Gooding W, Whiteside TL, Ferrone S, et al. Defects in the human leukocyte antigen class I antigen processing machinery in head and neck squamous cell carcinoma: association with clinical outcome. Clin Cancer Res 2005 Apr 1;11(7):2552-60.

(39) Murakami Y, Kanda K, Yokota K, Kanayama H, Kagawa S. Prognostic significance of immuno-proteosome subunit expression in patients with renal-cell carcinoma: a preliminary study. Mol Urol 2001;5(3):113-9.

(40) Belich MP, Glynne RJ, Senger G, Sheer D, Trowsdale J. Proteasome components with reciprocal expression to that of the MHC-encoded LMP proteins. Curr Biol 1994 Sep 1;4(9):769-76.

(41) Frankel A, Man S, Elliott P, Adams J, Kerbel RS. Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. Clin Cancer Res 2000 Sep;6(9):3719-28.

(42) Cusack JCJr, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J, et al. Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. Cancer Res 2001 May 1;61(9):3535-40.

(43) Khan AN, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. Cancer Immunol Immunother 2008 May;57(5):647-54.

(44) Rolland P, Deen S, Scott I, Durrant L, Spendlove I. Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer. Clin Cancer Res 2007 Jun 15;13(12):3591-6.

(45) Ogino T, Shigyo H, Ishii H, Katayama A, Miyokawa N, Harabuchi Y, et al. HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker. Cancer Res 2006 Sep 15;66(18):9281-9.

(46) Leffers N, Gooden MJ, De Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, et al. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. Cancer Immunol Immunother. In press 2008.

# Chapter 5

## Identification of genes and pathways associated with cytotoxic T-lymphocyte infiltration of serous ovarian cancer



Leffers N*, Fehrmann RSN*, Gooden MJM, Schulze URJ, Hollema H, Boezen HM, Daemen T, de Jong S, Nijman HW, van der Zee AGJ.

* Both authors contributed equally

*Manuscript in preparation*

## ABSTRACT

### Background

Although it has been well-established that tumour-infiltrating lymphocytes (TIL) are predictors of disease-specific survival in ovarian cancer, it is largely unknown what factors contribute to the presence of these lymphocytes. The aim of this study was to evaluate which genes and pathways contribute to the presence of cytotoxic T-lymphocytes in advanced stage serious ovarian cancer.

### Methods

Samples of 25 low TIL tumours and 24 high TIL tumours from advanced stage serous ovarian cancer patients were arrayed by means of oligonucleotide microarrays. Parametric T-testing was used to evaluate differences in gene expression between low and high TIL tumour samples. Gene set enrichment analysis was performed to identify selectively enriched biological pathways in low and high TIL samples. Prognostic influence of identified genes and pathways was validated in 157 previously profiled late stage serous ovarian cancer patients. With immunohistochemistry, association of selected genes from identified pathways with lymphocytes was validated.

### Results

A total of 320 genes and 23 pathways were associated with the presence of lymphocytes ($P < 0.05$). Of these, 54 genes and 8 pathways were also associated with disease-specific survival in our validation cohort. Genes positively associated with $CD8^+$ TIL generally correlated with improved survival. Confirming microarray results, immunohistochemical evaluation showed strong correlations between MHC class I and II membrane expression, part of the identified antigen processing and presentation pathway, and number of $CD8^+$ T-lymphocytes present.

### Conclusion

Gene expression profiling and pathway analyses are valuable tools to obtain more understanding of tumour characteristics influencing lymphocyte recruitment in advanced stage serous ovarian cancer. Identified genes and pathways need to be further investigated for their possible value as therapeutic targets.

## INTRODUCTION

Although not the most frequently diagnosed type of gynaecological malignancy, epithelial ovarian cancer is the most common cause of death from gynaecological malignancies (1). Five year survival rates for ovarian cancer patients do not exceed 40%. The high mortality is best attributed to the absence of specific symptoms combined with the lack of reliable screening methods, which prevent diagnosis in early stages of disease in the majority of patients. Treatment generally consists of cytoreductive surgery followed by platinum and taxane containing chemotherapy. Classic prognostic factors are stage of disease at diagnosis, histological tumour type and grade, residual disease after primary surgery and response to chemotherapy (2). An increasing body of evidence suggests that next to these established prognostic factors, the presence of tumour-infiltrating lymphocytes (TIL) also independently contributes to prognosis (3-5). These TIL are generally considered a reflection of anti-tumour immunity. It is largely unknown why TIL are present in high numbers in some tumours and largely absent in others, although it has recently been demonstrated that endothelial factors and chemokines secreted by the tumour may play an important role (6;7). The existence of an anti-tumour immune repertoire in a selection of patients forms the rationale for the development of cancer immunotherapy. Although immunotherapy strategies generally induce potent peripheral immune responses in ovarian cancer patients, clinical responses have so far been disappointing (8;9). The combination of targeted agents that enhance lymphocyte recruitment to tumour sites with these immunotherapy strategies might be a lucrative approach to obtain clinical responses to immunotherapy. For instance, the *in vivo* addition of BQ-788, an endothelin B receptor antagonist, to previously immunogenic, but clinically ineffective immunization strategies, resulted in enhanced homing of lymphocytes to tumours as well as improved clinical responses (6).

To investigate what tumour factors contribute to the recruitment of lymphocytes, we analyzed which genes and pathways were associated with the presence of tumour-infiltrating cytotoxic T-lymphocytes in a homogeneous group of 49 advanced stage serous ovarian cancer patients previously profiled at our institute as part of a large study (10). The prognostic value of identified genes and pathways was subsequently validated on all 157 previously profiled late stage serous ovarian cancer patients (10). Furthermore, immunohistochemical staining of tissue microarrays was performed to validate findings.

## METHODS

### Patients

The presence of tumour-infiltrating T-lymphocytes was previously evaluated by our group in 306 ovarian cancer patients (5). For this study, we selected advanced stage serous ovarian cancer patients with few (≤ 5 per 0.283 $mm^2$ of tumour) or many (≥ 8 per 0.283 $mm^2$ of tumour) cytotoxic T-lymphocytes who were also included in the previously published microarray study (10).

Patients were treated at the University Medical Center Groningen by a gynaecological oncologist and staged according to FIGO classification (11). Tumours were graded and classified according to WHO criteria by a gynaecological pathologist (12). Adjuvant chemotherapy generally consisted of different platinum-based treatment regimens. Response to chemotherapy was evaluated according to WHO criteria (13). After treatment, patients were followed-up for at least 10 years with gradually increasing intervals. Informed consent was obtained for the collection and storage of tumour samples in a tissue bank for future research. Information on clinicopathological characteristics and follow-up of patients was obtained from a computerized database in which information of all patients with epithelial ovarian cancer treated at our institute is prospectively recorded. For the present study, relevant data were retrieved into a separate anonymous database. In this separate database, patient identity was protected by study-specific, unique patient codes. In case of uncertainties with respect to clinicopathological and follow-up data, the larger databases could only be checked through two data managers who have daily responsibilities for the larger database, thereby ascertaining the protection of patients' identity. According to Dutch law no approval from our IRB was needed.



### Microarray analysis

We selected 49 advanced stage serous ovarian cancer patients, based on the relative absence or presence of TIL, who were previously profiled as part of a larger study (10). In brief, after RNA extraction and amplification, samples were hybridized to two-colour 70-mer oligonucleotide microarrays (~35.000 Operon v3.0 probes). All samples were hybridized at least twice and samples were loaded using a random design to prevent systematic biases (14-16). Arrays were scanned with the Affymetrix GMS428 (Affymetrix, Santa Clara, CA). Expression values were calculated by Bluefuse software (BlueGnome, Cambridge, UK). Operon v3.0 probe identifiers were converted to official gene symbols using probe annotations provided by the Netherlands Cancer Institute (http://microarrays.nki.nl/services/blastdata.html). Only oligonucleotides specifically responding with a single hit on a gene during a BLAST search were used. Expression values of multiple probes targeting a single gene were averaged, resulting in a total of 15909 distinct genes. Subsequently, expression data of the multiple hybridizations per tumour sample were averaged. Microarray data of the previous, larger study from which our patients were selected are available at http://www.ncbi.nlm.nih.gov/geo/ under number GSE13876.

**Class comparison between low TIL and high TIL samples**

The BRB Array Tools 3.6.0 software package, developed by the Biometrich Research Branch of the US National Cancer Institute, was used for class comparison between low TIL and high TIL samples. Differentially expressed genes were identified using a paired t-test (threshold $P < 0.05$).

**Gene set enrichment analysis**

As it is unclear whether large differences in expression of a single gene are biologically more relevant than more subtle, though coordinated differences in a set of genes belonging to a single biological pathway, we performed gene set enrichment analysis (GSEA). Expression data of all 15909 genes were compared against functional gene sets to determine whether any of these sets were enriched in samples containing many or few TIL. The comparison was performed using 340 gene sets reported in two databases (174 sets from Biocarta: http://www.biocarta.com; 166 sets from Kyoto Encyclopedia of Genes and Genomes database (KEGG); http://www.genome.jp/kegg/). Statistical significance of enrichment was determined using an empirical gene-based permutation test using 1000 permutations. Gene sets with an enrichment p-value <0.05 are reported. We also calculated false discovery rates (FDR) for each functional gene set, which represent the estimated probability that a given enrichment score represents a false positive finding. We only report gene sets with an FDR <0.25. With such a FDR, results are likely to be valid at least three out of four times, which is considered a suitable cut-off for the generation of interesting hypotheses (17). GSEA was executed with GSEA 2.0 software package (Broad Institute of MIT and Harvard, Cambridge, MA).

**Leading-edge subset analysis**

The leading-edge subset is defined as the subset of genes in a functional gene set that appears in the ranked list of 15.909 genes at, or before, the point where the running enrichment score reaches its maximum deviation from zero. The genes within this subset can be interpreted as the most important in the enrichment of the functional gene set. Leading-edge subsets were defined for all statistically enriched function gene sets ($P < 0.05$). Subsequently, overlap between leading-edge subsets from significantly enriched functional gene sets identified in the different databases was determined to discover genes belonging to more than one leading-edge subset, i.e. possible key genes.

**Prognostic value of identified genes and pathways**

Genes identified as differentially expressed between high and low TIL containing samples were correlated with disease-specific survival in a large cohort of 157 late stage ovarian cancer patients previously profiled at our institution (10). The log expression levels of individual genes were entered into a univariate Cox proportional hazards regression model. Genes with a $P$ <0.05 were considered to be associated with disease-specific survival. Furthermore, GSEA was performed on the cohort of

157 ovarian cancers to evaluate associations between disease-specific survival and identified pathways.

**Immunohistochemistry**

Protein expression of selected enriched genes from identified pathways was evaluated by immunohistochemistry using tissue microarrays sections. Tissue microarrays were constructed from paraffin embedded tumour tissue obtained at primary debulking surgery, performed by the gynaecological oncologists from the University Medical Center Groningen between May 1985 and April 2003. The tissue microarrays contain four 0.6mm core biopsies from each of 361 patients. For the present study, tissue samples obtained at primary debulking surgery from 108 advanced stage serous ovarian cancer patients were analyzed, for whom staining of tumour-infiltrating lymphocytes and HLA-A and HLA-B/C has previously been performed and published (5;18). In addition, staining was performed for HLA-DP/DQ/DR (clone CR3/43, DAKO, Heverlee, Belgium). In brief, after dewaxing and rehydration, four µm sections were microwaved in 10mM citrate buffer pH 6.0 for antigen retrieval. Subsequently, sections were incubated overnight with the primary antibody (dilution 1:100). Sections were subsequently incubated with DAKO Envision+ (DAKO, Heverlee, Belgium). Antigen-antibody reactions were visualized with 3,3-diaminobenzidine. Tissue was counterstained with haematoxylin.



Sections were scored independently by two observers (M.G. / N.L.) unaware of clinicopathological characteristics and TIL status of patients. A semi-quantitative quality control system was used taking into account both intensity of staining and percentage of positive tumour cells as previously described (18). The sum of both scores was used to identify three categories of expression: no expression (total score 0-2), positive expression in proportion of cells or weak expression in all cells (total score 3-6), and positive expression in majority of cells (total score 7-8). Only patients for whom at least two evaluable cores were available were included for further analysis.

Associations between $CD8^+$ TIL and protein expression of selected enriched genes in identified pathways were evaluated with the Jonckheere-Terpstra test (a form of the Kruskal-Wallis test which also tests for linearity). The Mann-Whitney U test was used to evaluate associations between $CD8^+$ TIL and well-known prognostic factors (i.e. age, FIGO stage, histological grade and residual tumour after primary debulking surgery). Disease-specific survival (DSS) was defined as date of surgery until date of death due to ovarian cancer or date of last follow-up. Differences in DSS based on protein expression levels were plotted using Kaplan-Meier survival curves and evaluated by log-rank tests. SPSS software package for Windows, version 16.0 (SPSS Inc., Chicago, USA) was used. $P$ values $< 0.05$ were considered statistically significant.

## RESULTS

### Patient characteristics

Microarray data of 49 advanced stage serous ovarian cancer patients with either few $CD8^+$ TIL (n=25) or many $CD8^+$ TIL (n=24) were evaluated. Clinical and pathological characteristics as well as tumour percentage of the samples used for microarray did not differ between patients with few or many $CD8^+$ TIL (table 1). Median disease-specific survival was higher for patients with high $CD8^+$ TIL (log rank test p=0.025).

### Differential expression of genes and biological pathway analysis

Comparison of expression levels of all 15909 genes showed differential expression of 320 genes between tumours containing few and many $CD8^+$ TIL (*P* value < 0.05). Differences between low and high $CD8^+$ TIL samples were small (Supplementary Table 1). In view of these small differences, we subsequently evaluated whether coordinated differences in genes belonging to a single biological pathway existed rather than differences in expression levels of single genes. Fourteen pathways in BIOCARTA and 8 pathways in KEGG ($p<0.05$, FDR < 0.25) were enriched in tumour samples with many $CD8^+$ TIL, whereas only one pathway was enriched in tumour samples with few $CD8^+$ TIL (table 2). Interestingly, and conveniently serving as an internal control, one of the pathways enriched in high $CD8^+$ TIL tumours, was the CTL mediated immune response pathway. We subsequently performed leading-edge subset analysis to identify key regulatory genes common to the enriched pathways (table 3), which among others identified several genes encoding for HLA molecules.

### Impact on disease specific survival

Univariate survival analysis was performed to assess the prognostic value of the 320 genes identified as differentially expressed between high and low TIL tumours, using 157 late stage serous ovarian cancer patients previously profiled at our institute (10). A significant association with disease-specific survival was observed for 54 genes (table 4). Of the 23 genes associated with high TIL tumours, 21 were associated with improved survival. Conversely, 27 of 31 genes associated with low TIL tumours were associated with decreased survival (figure 2). Next, we performed GSEA to identify which pathways were associated with disease specific survival. Eight pathways associated with the presence of $CD8^+$ TIL, such as the antigen processing and presentation pathway, were also positively associated with disease-specific survival (table 2).

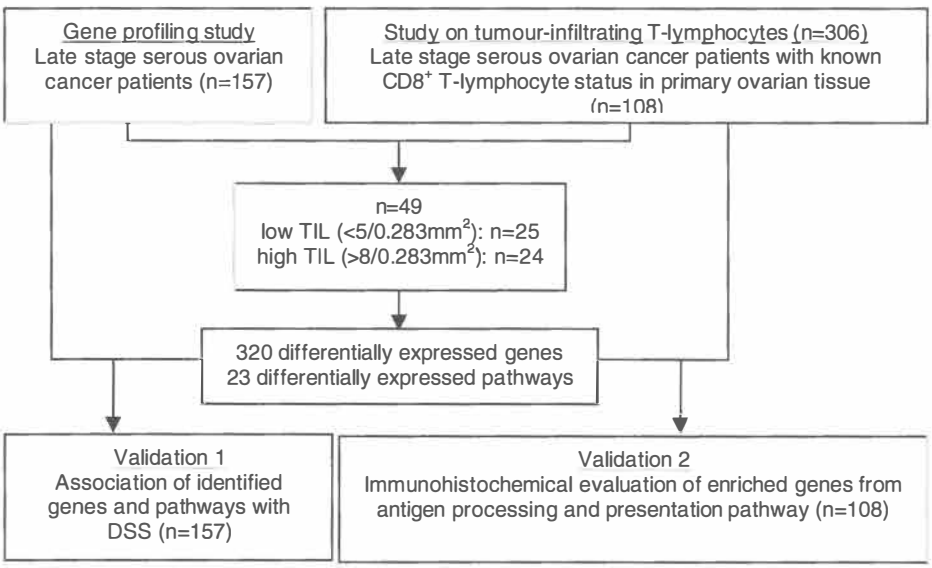


**Fig. 1** Flow chart illustrating patient selection and validation. Index and validation patients were selected from previous studies at our institute investigating prognostic impact of gene expression (10) and tumour-infiltrating lymphocytes in ovarian cancer patients (5).



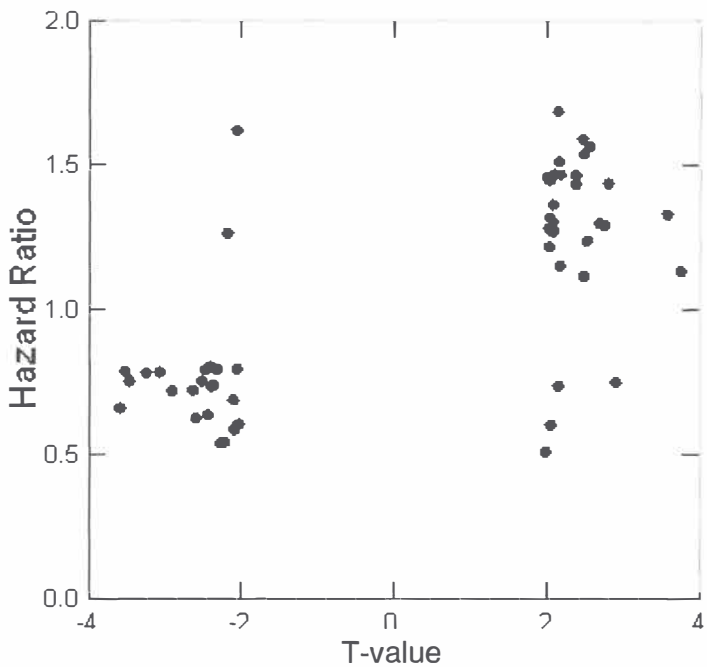


**Fig. 2** Scatterplot illustrating clustering of differentially expressed genes with survival. Genes with T-value <0.0 are differentially expressed in high TIL tumours, genes with T-value >0.0 are differentially expressed in low TIL tumours. Hazard ratio < 1.0 increased survival; hazard ratio > 1.0 worse survival

### Immunohistochemical validation

Based on GSEA and leading-edge subset analysis, which showed that the presence of tumour-infiltrating lymphocytes was associated with expression of MHC class I and II genes as part of the antigen processing and presentation pathway, we evaluated immunohistochemical staining of HLA-A, HLA-B/C and HLA-DP/DQ/DR in 108 advanced stage serous ovarian cancer patients for whom information on TIL was available, part of which was previously published for a larger patient cohort (5;18). Partial or total loss of HLA-A and HLA-B/C was observed in 70.4% resp. 62.0% of patients, whereas HLA-DP/DQ/DR up-regulation was observed in 68.5% of patients. Increasing levels of MHC class I and II protein expression strongly correlated with increased numbers of $CD8^+$ T-lymphocytes (table 5). However, no association was observed between expression of HLA-A, HLA-B/C and HLA-DP/DQ/DR with disease-specific survival ($P = 0.114$, $P = 0.599$, $P = 0.692$ respectively).

**Table 1** Clinicopathological characteristics and survival data of patients with known $CD8^+$ TIL status

| | Patients in Microarray Analyses | | | | | IHC validation set | |
|---|---|---|---|---|---|---|---|
| | Low TIL (n=25) | | High TIL (n=24) | | *P** | (n=108) | |
| *Age (years)* | | | | | | | |
| Mean (SD) | 62.4 | (14.5) | 60.8 | (12.9) | n.s.* | 60.2 | (13.2) |
| *DSS (months)* | | | | | | | |
| Median (95%CI) | 10.8 | (8.7-13.0) | 19.4 | (1.0-37.7) | 0.025 | 19.9 | (8.4-31.3) |
| *FIGO Stage* | | | | | | | |
| Stage III | 19 | (76.0%) | 20 | (83.3%) | n.s. | 87 | (80.6%) |
| Stage IV | 6 | (24.0%) | 2 | (16.7%) | | 21 | (19.4%) |
| *Tumour Grade* | | | | | | | |
| Grade I | 1 | (4.0%) | 2 | (8.3%) | n.s. | 6 | (5.6%) |
| Grade II | 10 | (40.0%) | 5 | (20.8%) | | 28 | (25.9%) |
| Grade III / undifferentiated | 13 | (52.0%) | 16 | (66.7%) | | 67 | (62.0%) |
| Missing | 1 | (4.0%) | 1 | (4.2%) | | 7 | (6.5%) |
| *Residual disease* | | | | | | | |
| < 2 cm | 5 | (20.0%) | 6 | (25.0%) | n.s. | 33 | (30.6%) |
| >= 2 cm | 20 | (80.0%) | 16 | (66.7%) | | 66 | (61.1%) |
| Missing | - | - | 2 | (8.3%) | | 9 | (8.3%) |
| *Type of chemotherapy* | | | | | | | |
| No chemotherapy | 3 | (12.0%) | 1 | (4.2%) | n.s. | 6 | (5.6%) |
| Platinum containing | 11 | (44.0%) | 12 | (50.0%) | | 41 | (38.0%) |
| Platinum & taxane containing | 8 | (32.0%) | 8 | (33.3%) | | 48 | (44.4%) |
| Other | 2 | (8.0%) | 3 | (12.5%) | | 10 | (9.3%) |
| Missing | 1 | (4.0%) | - | - | | 3 | (2.8%) |
| *$CD8^+$* | | | | | | | |
| median (IQR) | 1.0 | (0.5-2.5) | 24.0 | (16.0-62.5) | <0.001 | 8.0 | (2.0-22.8) |
| *% tumour in microarray sample* | | | | | | | |
| median (IQR) | 63 | (50.0-80.0) | 70 | (52.5-78.0) | n.s. | | |

DSS = disease-specific survival; FIGO = International Federation of Gynaecology and Obstetrics; SD = standard deviation; CI = confidence interval; IHC = immunohistochemistry; * n.s. = not significant, i.e. $P \geq 0.05$

**Table 2** Enriched pathways identified by gene set enrichment analysis using pathway definitions from BIOCARTA and KEGG

| Pathway | database | P value | FDR* | Enriched in |
|---|---|---|---|---|
| Ribosome pathway | KEGG | 0.0 | 0.0 | Low TIL |
| Antigen processing and presentation# | KEGG | 0.0 | 0.0 | High TIL |
| Type I diabetes mellitus | KEGG | 0.0 | 0.006 | High TIL |
| Toll like receptor signalling pathway | KEGG | 0.0 | 0.055 | High TIL |
| Hematopoietic cell lineage# | KEGG | 0.0 | 0.060 | High TIL |
| Cytokine cytokine receptor interaction# | KEGG | 0.0 | 0.104 | High TIL |
| Cell adhesion molecules# | KEGG | 0.0 | 0.137 | High TIL |
| Citrate cycle | KEGG | 0.013 | 0.156 | High TIL |
| Reductive carboxylate cycle | KEGG | 0.038 | 0.142 | High TIL |
| CTL pathway# | BIOCARTA | 0.0 | 0.051 | High TIL |
| COMP pathway | BIOCARTA | 0.0 | 0.008 | High TIL |
| MITOCHONDRIA pathway | BIOCARTA | 0.0 | 0.120 | High TIL |
| D4GDI pathway | BIOCARTA | 0.005 | 0.105 | High TIL |
| AMI pathway | BIOCARTA | 0.005 | 0.225 | High TIL |
| DC pathway# | BIOCARTA | 0.007 | 0.215 | High TIL |
| TALL1 pathway# | BIOCARTA | 0.010 | 0.201 | High TIL |
| ATRBRCA pathway | BIOCARTA | 0.017 | 0.205 | High TIL |
| CSK pathway | BIOCARTA | 0.024 | 0.195 | High TIL |
| CASPASE pathway | BIOCARTA | 0.029 | 0.183 | High TIL |
| STEM pathway | BIOCARTA | 0.037 | 0.230 | High TIL |
| T CYTOTOXIC PATHWAY# | BIOCARTA | 0.038 | 0.198 | High TIL |
| ATM pathway | BIOCARTA | 0.038 | 0.198 | High TIL |
| LAIR pathway | BIOCARTA | 0.046 | 0.231 | High TIL |

*FDR: False discovery rate; #Also associated with improved survival in 157 previously profiled advanced stage ovarian cancer patients (10).

**Table 3** Results of leading edge analysis identifying genes common to enriched pathways

| KEGG | | BIOCARTA | |
|---|---|---|---|
| Gene symbol | N gene sets | Gene symbol | N gene sets |
| HLA-A | 4 | CD3D | 5 |
| HLA-B | 4 | ITGBB2 | 3 |
| HLA-DRB1 | 4 | CD3E | 4 |
| HLA-DRB2 | 4 | HLA-DRB1 | 3 |
| IL1B | 4 | CFS2 | 3 |
| TNFA1 | 4 | IL5 | 3 |
| HLA-DQB1 | 3 | IL8 | 3 |
| HLA-DQB2 | 3 | CD8A | 3 |
| HLA-F | 3 | CYCS | 3 |
| CD8A | 3 | GZMB | 3 |
| NFKB1A | 3 | PRF1 | 3 |
| RELA | 3 | RELA | 3 |
| IKBKB | 3 | | |
| PIK3R3 | 3 | | |
| CSF2 | 3 | | |
| IL8 | 3 | | |
| CCL5 | 3 | | |
| CD40 | 3 | | |

## DISCUSSION

Although it has been well-established that tumour-infiltrating lymphocytes are predictors of disease-specific survival in ovarian cancer, it is largely unknown what factors contribute to the presence of these lymphocytes. By comparing gene expression profiles of 25 tumours containing few and 24 tumours containing many $CD8^+$ T-lymphocytes, we identified 320 genes differentially expressed by primary tumours of late stage serous ovarian cancer patients. Additionally, for 54 of these genes an association with survival was observed in a large validation cohort containing 157 advanced stage serous ovarian cancer patients. Genes connected to high TIL tumours were associated with improved survival. With GSEA, next to pathways merely reflecting presence of lymphocytes, several pathways were identified to be associated with the (lack of) TIL, some of which were also associated with survival in our validation set. Lastly, the association of a number of genes from the enriched antigen processing and presentation pathway with the presence of $CD8^+$ T-lymphocytes was confirmed by immunohistochemistry.

Our study illustrates that gene expression profiling is a valuable approach to elucidate what tumour-cell characteristics contribute to or impede recruitment of lymphocytes into serous ovarian cancer. However, a problem inherent to the design of our study is the impossibility to discern what genes were expressed by tumour cells and what signal derived from tumour-infiltrating lymphocytes themselves. This is substantiated by the fact that some of the pathways found to be enriched in high TIL tumours are lymphocyte-specific pathways, e.g. T cytotoxic pathway, an observation which could also be regarded to validate the immunohistochemical evaluation of $CD8^+$ T-cell count used for patient selection. To avoid the signal from lymphocytes, one could try to profile only tumour cells. This could be accomplished by microdissection of tumour cells, followed by profiling of a single or only a limited number of tumour cells (19). However, as with decreasing cell numbers RNA yield also reduces, such assays depend heavily on mRNA amplification. Especially genes with low numbers of transcripts may be underrepresented after amplification and thus not identified in subsequent profiling studies (20). For the present study, we therefore decided that, as the percentage of tumour cells did not differ between samples with few and many TIL, it was safe to assume that differences in non-lymphocyte restricted genes and pathways reflected differences in gene expression by tumour cells. When analyzing results one should keep in mind that genome-wide gene profiling is to be regarded a hypothesis-generating technique.

Ultimately, our study was intended not only to establish what tumour factors contribute to lymphocyte recruitment but also to discover putative factors that might enhance clinical results of immunotherapy for ovarian malignancies by improving lymphocyte recruitment when targeted. In this respect, one of the interesting genes identified as differentially expressed between high TIL and low TIL tumours and associated with disease-specific survival of late stage serous ovarian cancer patients

**Table 4** Genes differentially expressed between high and low TIL tumours that are associated with disease-specific survival in a large cohort of 157 advanced stage serous ovarian carcinomas

| Gene symbol | Description | T | Parametric p-value TIL | Parametric p-value DSS | HR | Fold change high/low TIL |
|---|---|---|---|---|---|---|
| ENDOG | endonuclease g | -3.58818 | 0.000469 | 0.007736 | 0.657 | 1.057368 |
| APOL6 | apolipoprotein l. 6 | -3.51777 | 0.000588 | 0.016474 | 0.784 | 1.061243 |
| LOC144817 | hypothetical protein loc144817 | -3.4572 | 0.000725 | 0.007082 | 0.751 | 1.054609 |
| TNFRSF11B | tumor necrosis factor receptor superfamily. member 11b (osteoprotegerin) | -3.2365 | 0.001533 | 0.029561 | 0.778 | 1.076219 |
| CCL5 | chemokine (c-c motif) ligand 5 | -3.05761 | 0.002678 | 0.008189 | 0.78 | 1.06113 |
| SMARCD3 | swi/snf related. matrix associated. actin dependent regulator of chromatin. subfamily d. member 3 | -2.89433 | 0.004415 | 0.012281 | 0.715 | 1.044497 |
| HLA-DQB2 | major histocompatibility complex. class ii. dq beta 1 | -2.62341 | 0.009683 | 0.025419 | 0.718 | 1.036787 |
| P2RY2 | purinergic receptor p2y. g-protein coupled. 2 | -2.58765 | 0.010682 | 0.04794 | 0.622 | 1.062139 |
| HTATIP2 | hiv-1 tat interactive protein 2. 30kda | -2.50033 | 0.013646 | 0.023893 | 0.749 | 1.04496 |
| ITGB4 | integrin. beta 4 | -2.4564 | 0.015268 | 0.02019 | 0.788 | 1.042571 |
| BRSK1 | br serine/threonine kinase 1 | -2.4344 | 0.016188 | 0.001032 | 0.634 | 1.043443 |
| CD74 | cd74 antigen (invariant polypeptide of major histocompatibility complex. class ii antigen-associated) | -2.39153 | 0.018114 | 0.048429 | 0.798 | 1.0387 |
| IRF1 | interferon regulatory factor 1 | -2.38234 | 0.018578 | 0.018766 | 0.731 | 1.039269 |
| CARD9 | caspase recruitment domain family. member 9 | -2.34654 | 0.020354 | 0.012632 | 0.733 | 1.053982 |
| GBP5 | guanylate binding protein 5 | -2.29852 | 0.023022 | 0.026438 | 0.789 | 1.053155 |
| OR4K1 | olfactory receptor. family 4. subfamily k. member 1 | -2.25082 | 0.025956 | 0.027515 | 0.535 | 1.043061 |
| IDH3A | isocitrate dehydrogenase 3 (nad+) alpha | -2.19777 | 0.029762 | 0.004543 | 0.539 | 1.042238 |
| RARRES3 | retinoic acid receptor responder (tazarotene induced) 3 | -2.17032 | 0.031671 | 0.027127 | 1.26 | 1.039329 |
| GUCY2F | guanylate cyclase 2f. retinal | -2.09159 | 0.038394 | 0.030464 | 0.683 | 1.064628 |
| C1orf151 | chromosome 1 open reading frame 151 | -2.08091 | 0.039363 | 0.000145 | 0.582 | 1.037702 |
| PUM2 | pumilio homolog 2 (drosophila) | -2.04334 | 0.042905 | 0.037206 | 1.617 | 1.026572 |
| SUSD3 | sushi domain containing 3 | -2.04205 | 0.043025 | 0.0087 | 0.791 | 1.041484 |
| GRM3 | glutamate receptor. metabotropic 3 | -2.02279 | 0.045086 | 0.007333 | 0.602 | 1.037417 |
| RP9 | retinitis pigmentosa 9 (autosomal dominant) | 1.979686 | 0.049732 | 0.02947 | 0.506 | 0.970948 |
| TPM2 | tropomyosin 2 (beta) | 2.01335 | 0.046075 | 0.002014 | 1.459 | 0.968061 |
| CREB3L4 | camp responsive element binding protein 3-like 4 | 2.024371 | 0.044851 | 0.024523 | 1.448 | 0.969375 |
| ADFP | adipose differentiation-related protein | 2.028392 | 0.044437 | 0.004516 | 1.279 | 0.954856 |

*Table 4, continued*

| | | | | | | |
|---|---|---|---|---|---|---|
| ZIC1 | zic family member 1 (odd-paired homolog. drosophila) | 2.034892 | 0.043757 | 0.024456 | 1.214 | 0.941812 |
| TCF4 | transcription factor 4 | 2.038027 | 0.043448 | 0.041311 | 1.317 | 0.965471 |
| RKHD1 | ring finger and KH domain containing 1 | 2.054802 | 0.041812 | 0.026765 | 0.6 | 0.971522 |
| AKAP12 | a kinase (prka) anchor protein (gravin) 12 | 2.05607 | 0.041754 | 0.020387 | 1.448 | 0.965955 |
| CENPF | centromere protein f. 350/400ka (mitosin) | 2.079257 | 0.039457 | 0.017257 | 1.28 | 0.955777 |
| TF | transferrin | 2.085059 | 0.039044 | 0.019715 | 1.302 | 0.95165 |
| MARCKS | myristoylated alanine-rich protein kinase c substrate | 2.085433 | 0.038849 | 0.01913 | 1.361 | 0.967259 |
| FBN3 | fibrillin 3 | 2.08694 | 0.038733 | 0.008793 | 1.266 | 0.942574 |
| TSPAN13 | tetraspanin 13 | 2.102597 | 0.037447 | 0.00351 | 1.465 | 0.962836 |
| C1orf85 | chromosome 1 open reading frame 85 | 2.149681 | 0.033301 | 0.009093 | 1.684 | 0.969927 |
| NT5C2 | 5'-nucleotidase. cytosolic ii | 2.151955 | 0.033136 | 0.042652 | 0.735 | 0.959484 |
| DAD1 | defender against cell death 1 | 2.163076 | 0.032559 | 0.039308 | 1.509 | 0.971288 |
| COL11A1 | collagen. type xi. alpha 1 | 2.180716 | 0.03094 | 0.008437 | 1.149 | 0.926402 |
| GLT25D1 | glycosyltransferase 25 domain containing 1 | 2.183846 | 0.030707 | 0.011982 | 1.464 | 0.966298 |
| GMFB | glia maturation factor. beta | 2.381146 | 0.018725 | 0.011898 | 1.432 | 0.962138 |
| SNRPE | small nuclear ribonucleoprotein polypeptide e | 2.38174 | 0.018656 | 0.012262 | 1.462 | 0.983812 |
| ZNF281 | zinc finger protein 281 | 2.473985 | 0.014571 | 0.023546 | 1.587 | 0.957854 |
| FABP4 | fatty acid binding protein 4. adipocyte | 2.485825 | 0.014192 | 0.025061 | 1.11 | 0.908835 |
| SAE2 | SUMO1 activating enzyme subunit 2 | 2.489129 | 0.014051 | 0.018709 | 1.535 | 0.965225 |
| BASP1 | brain abundant. membrane attached signal protein 1 | 2.533806 | 0.012459 | 0.032678 | 1.235 | 0.94384 |
| ARMCX3 | armadillo repeat containing. x-linked 3 | 2.563703 | 0.011458 | 0.006661 | 1.562 | 0.94847 |
| CPVL | carboxypeptidase. vitellogenic-like | 2.690174 | 0.008038 | 0.048239 | 1.295 | 0.957396 |
| FMOD | fibromodulin | 2.76574 | 0.006462 | 0.012624 | 1.289 | 0.945678 |
| C3orf59 | chromosome 3 open reading frame 59 | 2.822682 | 0.005501 | 0.022204 | 1.433 | 0.950026 |
| PXDN | peroxidasin homolog (drosophila) | 2.920898 | 0.004082 | 0.049685 | 0.746 | 0.94662 |
| MEST | mesoderm specific transcript homolog (mouse) | 3.590373 | 0.000463 | 0.002053 | 1.328 | 0.940516 |
| HMGA2 | high mobility group at-hook 2 | 3.770553 | 0.00024 | 0.031783 | 1.128 | 0.886346 |

TIL – tumour-infiltrating lymphocytes; DSS – disease specific survival; HR – hazard ratio

is interferon regulatory factor 1 (IRF-1). Recently, IRF-1 was reported to be a positive prognostic factor in ovarian and colorectal cancer (21;22) and was found to be associated with CTL infiltration in ovarian cancer (23). Binding of IFN-γ to the IFN-γ receptor leads to up-regulation of IRF-1, which in turn results in 1) induction of IFN-γ inducible genes, such as the TAP1, LMP2 and $\beta_2$-microglobulin genes of the MHC class I dependent pathway, as well as 2) activation of CIITA, a critical transcription factor for MHC class II gene expression. IRF-1 thus facilitates recognition of tumour cells by immune cells, ultimately resulting in an IFN-γ dependent positive feedback loop. Correspondingly, in high TIL tumours we observed differential expression of several MHC class I and II genes, all part of the selectively activated antigen processing and presentation pathway. Immunohistochemical evaluation confirmed the positive association of intra-tumoural cytotoxic T-cells with surface expression of MHC class I and II molecules HLA-A, HLA-B/C and HLA-DP/DQ/DR. Although several MHC class II alleles were differentially expressed in high TIL tumours, no association was found between HLA-DP/DQ/DR membrane expression and survival. Moreover, only HLA-DQB2, a virtually non-polymorphic gene was associated with disease-specific survival in our large validation cohort (24). As it encodes six thus far unknown putative proteins, the association of HLA-DQB2 with TIL and survival is intriguing and deserves further investigation.



While we previously reported decreased survival for ovarian cancer patients in association with HLA-B/C down-regulation, an association with survival was observed neither at mRNA nor protein level in the present study (25). The difference in prognostic impact observed in the present study could be explained by differences in study population and/or size. In the present study a homogeneous population of 108 late stage serous ovarian cancer patients was used for immunohistochemical validation. Renewed analysis of the previously published data (25) using only late stage serous ovarian cancer patients (n=151), did not yield a correlation of HLA-B/C expression with disease specific survival either (data not shown).

To our knowledge, only one study attempting a better understanding of mechanisms underlying tumour-infiltrating cytotoxic T-cells in serous ovarian cancer by gene profiling of tumour samples has previously been published (23). In this study profiling 38 high-grade advanced stage ovarian carcinomas, 81 genes were associated with $CD8^+$ T-cell infiltrate. Only two of these genes, IRF-1 and CXCR6, were also found to be differentially expressed in our cohort of 49 serous advanced stage ovarian cancer patients ($P$ = 0.018578 resp. $P$ = 0.035424). Several possible explanations for the lack of concordance in identified genes exist. Technique-specific issues, including choice of microarray platform and randomisation of samples on arrays, may impede overlap in results (26). Furthermore, differences in patient population exist between the two studies, e.g. only high-grade tumours vs. low and high-grade tumours in our study. An additional difficulty, inherent to microarray studies, is the use of small patient cohorts to evaluate large numbers of potential predictors of lymphocyte

recruitment. This raises the likelihood of finding distinctive patterns based on chance rather than on biology, a phenomenon called overfitting (27).

Despite these challenges, expression of IRF-1, described above, and CXCR6 was positively associated with the presence of $CD8^+$ T-lymphocytes in both studies. Recently, it was established that both the chemokine receptor CXCR6 and its ligand CXCL16 are not only expressed by immune cells, but also by carcinomas (28). Moreover, radiation was shown to recruit lymphocytes to carcinomas through the release of CXCL16 by tumour cells (29). Whether expression of CXCR6 is similarly induced by radiation and also influences lymphocyte attraction remains to be investigated.

Not only was there a discrepancy between identified genes between our and Callahan's study (23), we also found a substantial difference in genes and pathways associated with survival between the present and our previous study (10). An important reason for this divergence is a difference in approach. Whereas the association with survival was the primary focus of our previous study, the current study was designed to discover genes and pathways which might be linked to lymphocyte recruitment. Only identified genes and pathways were subsequently evaluated for association with survival. A second, though related reason is the fact that for the present study we were less stringent in selection of genes ($P < 0.05$) since we were primarily interested in associations with lymphocyte recruitment rather than survival, while for the 86-gene profile only genes meeting a $P < 0.001$ were selected (10). Thus only two genes associated with survival in the current study were also part of the 86-gene profile, i.e. BRSK1 and C1orf151. Neither gene has so far been further investigated to explain its prognostic impact and/or role in lymphocyte recruitment.



**Table 5** Association of HLA protein expression evaluated by immunohistochemistry with $CD8^+$ tumour-infiltrating lymphocytes in serous advanced stage ovarian cancer

| | | $CD8^+$ T-lymphocytes | | | |
|---|---|---|---|---|---|
| | N (%) | Median | IQR | *Z* | *P* * |
| *HLA-A* | | | | | |
| Total loss | 27 (25.0) | 1.0 | 1.0-12.0 | 3.96 | **<0.001** |
| Partial loss | 49 (45.4) | 9.0 | 4.0-22.0 | | |
| Normal expression | 32 (29.6) | 14.0 | 4.8-55.3 | | |
| *HLA-B/C* | | | | | |
| Total loss | 23 (21.3) | 2.0 | 1.0-15.0 | 4.19 | **<0.001** |
| Partial loss | 44 (40.7) | 6.0 | 2.0-19.8 | | |
| Normal expression | 41 (38.0) | 16.0 | 5.5-30.5 | | |
| *HLA-DP/DQ/DR* | | | | | |
| No expression | 34 (31.5) | 2.0 | 1.0-11.0 | 3.36 | **0.001** |
| Upregulation | 52 (48.1) | 12.0 | 5.0-24.0 | | |
| Strong upregulation | 22 (20.4) | 14.5 | 4.5-43.0 | | |

IQR = interquartile range. * calculated by means of Jonckheere Terpstra test.

In summary, this study shows that gene expression profiling and pathway analysis are valuable strategies to obtain more insight into what tumour characteristics contribute to lymphocyte recruitment to advanced stage serous ovarian carcinomas. Identified genes and pathways need to be further validated and evaluated for their value as therapeutic target.

**REFERENCES**


(1) Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55:74-108.

(2) Crijns AP, Duiker EW, de Jong S, Willemse PH, van der Zee AG, de Vries EG. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer 2006; 16 Suppl 1:152-165.

(3) Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348:203-213.

(4) Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005; 102:18538-18543.

(5) Leffers N, Gooden MJ, de Jong RA, et al. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. Cancer Immunol Immunother 2009; 58:449-459.

(6) Buckanovich RJ, Facciabene A, Kim S, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med 2008; 14:28-36.

(7) Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004; 10:942-949.

(8) Hung CF, Wu TC, Monie A, Roden R. Antigen-specific immunotherapy of cervical and ovarian cancer. Immunol Rev 2008; 222:43-69.

(9) Leffers N, Daemen T, Helfrich W, et al. Antigen-specific active immunotherapy for ovarian cancer. Cochrane Database of Systematic Reviews 2009; *submitted*.

(10) Crijns AP, Fehrmann RS, de Jong S, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009; 6:e24.

(11) Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986; 25:383-385.

(12) Scully RE. Histological Typing of Ovarian Tumours. 2 ed. Berlin: Springer, 1999.

(13) World Health Organization. Handbook for Reporting Results of Cancer Treatment. Geneva: World Health Organization, 1979.

(14) Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nat Genet 2002; 32 Suppl:490-495.

(15) Hsu JC, Chang J, Wang T, Steingrimsson E, Magnusson MK, Bergsteinsdottir K. Statistically designing microarrays and microarray experiments to enhance sensitivity and specificity. Brief Bioinform 2007; 8:22-31.

(16) Kerr MK. Design considerations for efficient and effective microarray studies. Biometrics 2003; 59:822-828.

(17) Gu Q, Tan M, Sun Y. SAG/ROC2/Rbx2 is a novel activator protein-1 target that promotes c-Jun degradation and inhibits 12-O-tetradecanoylphorbol-13-acetate-induced neoplastic transformation. Cancer Res 2007; 67:3616-3625.

(18) Leffers N, Gooden MJ, Mokhova AA, et al. Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. Gynecol Oncol 2009; 113:256-263.

(19) Glanzer JG, Eberwine JH. Expression profiling of small cellular samples in cancer: less is more. Br J Cancer 2004; 90:1111-1114.

(20) Nygaard V, Holden M, Loland A, Langaas M, Myklebost O, Hovig E. Limitations of mRNA amplification from small-size cell samples. BMC Genomics 2005; 6:147.

(21) Zeimet AG, Reimer D, Wolf D, et al. Intratumoral interferon regulatory factor (IRF)-1 but not IRF-2 is of relevance in predicting patient outcome in ovarian cancer. Int J Cancer 2009; 124:2353-2360.

(22) Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313:1960-1964.

(23) Callahan MJ, Nagymanyoki Z, Bonome T, et al. Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer. Clin Cancer Res 2008; 14:7667-7673.

(24) Gaur LK, Heise ER, Thurtle PS, Nepom GT. Conservation of the HLA-DQB2 locus in nonhuman primates. J Immunol 1992; 149:2530.

(25) Leffers N, Lambeck AJA, de Graeff P, et al. Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. Gynecol Oncol 2008; 110:365-373.

(26) Draghici S, Khatri P, Eklund AC, Szallasi Z. Reliability and reproducibility issues in DNA microarray measurements. Trends Genet 2006; 22:101-109.

(27) Fehrmann RS, Li XY, van der Zee AG, et al. Profiling studies in ovarian cancer: a review. Oncologist 2007; 12:960-966.

(28) Meijer J, Ogink J, Kreike B, Nuyten D, de Visser KE, Roos E. The chemokine receptor CXCR6 and its ligand CXCL16 are expressed in carcinomas and inhibit proliferation. Cancer Res 2008; 68:4701-4708.

(29) Matsumura S, Wang B, Kawashima N, et al. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. J Immunol 2008; 181:3099-3107.

**Supplementary Table 1:** Differentially expressed genes between high and low TIL tumour samples

| Gene symbol | Parametric p-value | fold change (high TIL/low TIL) | Gene symbol | Parametric p-value | fold change (high TIL/low TIL) |
|---|---|---|---|---|---|
| LOC440871 | 7,43E-06 | 1,117251 | FCMD | 0,007847 | 1,05321 |
| C18orf16 | 4,97E-05 | 1,148209 | CPVL | 0,008038 | 0,957396 |
| SCG5 | 0,000131 | 0,923036 | NKG7 | 0,008153 | 1,065044 |
| IGJ | 0,000182 | 1,09965 | HLA-DRB5 | 0,00818 | 1,022035 |
| CMIP | 0,000183 | 1,067873 | EGLN3 | 0,008322 | 1,064923 |
| HMGA2 | 0,00024 | 0,886346 | RPP25 | 0,008593 | 1,056016 |
| PTEN | 0,000276 | 0,942375 | GPR149 | 0,008615 | 1,058556 |
| OCLN | 0,000363 | 1,079711 | HOXC6 | 0,008647 | 1,035913 |
| MEST | 0,000463 | 0,940516 | HRASLS2 | 0,009056 | 1,056497 |
| ENDOG | 0,000469 | 1,057368 | FAM114A1 | 0,009358 | 0,957671 |
| LOC650405 | 0,000571 | 1,091009 | DOK2 | 0,009436 | 1,037339 |
| APOL6 | 0,000588 | 1,061243 | HLA-DQB2 | 0,009683 | 1,036787 |
| LOC144817 | 0,000725 | 1,054609 | AFF2 | 0,009731 | 1,039478 |
| SERPINA1 | 0,00073 | 1,0625 | BMX | 0,00999 | 1,075003 |
| LCN2 | 0,001001 | 1,074102 | INTS1 | 0,010041 | 1,064488 |
| MYH10 | 0,00103 | 0,950461 | XPNPEP2 | 0,01009 | 1,076185 |
| BEXL1 | 0,001207 | 0,952302 | SFT2D1 | 0,010131 | 0,965259 |
| MFAP5 | 0,001248 | 0,929517 | PLS3 | 0,010246 | 0,940982 |
| CXCL9 | 0,001499 | 1,109033 | P2RY2 | 0,010682 | 1,062139 |
| TNFRSF11B | 0,001533 | 1,076219 | EPSTI1 | 0,010933 | 1,049835 |
| FST | 0,00155 | 0,929508 | ARMCX3 | 0,011458 | 0,94847 |
| INDO | 0,00155 | 1,078666 | DNAJC12 | 0,01184 | 0,926049 |
| HPR | 0,001764 | 1,089463 | RAB11A | 0,011922 | 1,038519 |
| C16orf24 | 0,001844 | 1,038406 | BASP1 | 0,012459 | 0,94384 |
| CDH2 | 0,002 | 0,953517 | SOSTDC1 | 0,012824 | 0,948313 |
| CD7 | 0,002024 | 1,084766 | NDN | 0,012829 | 0,948066 |
| CXCL10 | 0,002089 | 1,068514 | PLXDC2 | 0,013206 | 0,96359 |
| GBP1 | 0,002201 | 1,069424 | HTATIP2 | 0,013646 | 1,04496 |
| BEX1 | 0,002275 | 0,953609 | NGFRAP1 | 0,014045 | 0,931023 |
| OR7D2 | 0,002476 | 1,027473 | SAE2 | 0,014051 | 0,965225 |
| SAT1 | 0,002621 | 1,050012 | FABP4 | 0,014192 | 0,908835 |
| CCL5 | 0,002678 | 1,06113 | TCF7L2 | 0,014303 | 0,955611 |
| CTSH | 0,002974 | 1,0427 | HDAC2 | 0,014459 | 0,954354 |
| SCLY | 0,003134 | 1,064695 | ZNF281 | 0,014571 | 0,957854 |
| HRG | 0,003725 | 1,047394 | GDF15 | 0,014646 | 0,952374 |
| LOC652745 | 0,003735 | 1,039344 | SLC27A6 | 0,01489 | 1,058964 |
| BEX2 | 0,003791 | 0,959877 | CD2 | 0,014891 | 1,061113 |
| PXDN | 0,004082 | 0,94662 | EXOSC1 | 0,015039 | 0,963865 |
| SMARCD3 | 0,004415 | 1,044497 | RPL22L1 | 0,015255 | 0,956496 |
| ALCAM | 0,004481 | 0,947927 | ITGB4 | 0,015268 | 1,042571 |
| HP | 0,004653 | 1,091648 | TIMP3 | 0,015486 | 0,95625 |
| HLA-B | 0,005041 | 1,040748 | DUSP1 | 0,015651 | 0,949952 |
| OPLAH | 0,00505 | 1,071437 | BRSK1 | 0,016188 | 1,043443 |
| C3orf59 | 0,005501 | 0,950026 | RAB11FIP2 | 0,016538 | 0,953661 |
| ZDHHC14 | 0,005513 | 0,944562 | XPNPEP1 | 0,016544 | 0,959757 |
| TNFRSF9 | 0,005694 | 1,103201 | DKK1 | 0,01655 | 0,929787 |
| PTPRK | 0,005893 | 0,956806 | TREX1 | 0,016825 | 1,026756 |
| BST2 | 0,005894 | 1,043544 | TMSB10 | 0,016857 | 0,982603 |
| AGR2 | 0,005974 | 0,928906 | TLX3 | 0,017642 | 1,059934 |
| PSMB10 | 0,006215 | 1,05963 | FMO2 | 0,017694 | 0,930059 |
| FAHD1 | 0,006308 | 0,953147 | SERPINH1 | 0,017929 | 0,971745 |
| FMOD | 0,006462 | 0,945678 | SERPINE1 | 0,017941 | 0,932668 |
| IL2RG | 0,006827 | 1,075053 | CD74 | 0,018114 | 1,0387 |
| OR51I1 | 0,007021 | 1,043944 | ATP8B4 | 0,018262 | 1,050496 |
| TMOD1 | 0,007066 | 1,052378 | STXBP3 | 0,018296 | 1,040958 |
| CCL20 | 0,007176 | 1,082901 | HSD17B14 | 0,018508 | 0,945075 |
| TROVE2 | 0,007794 | 0,961688 | IRF1 | 0,018578 | 1,039269 |

| Gene symbol | Parametric p-value | fold change (high TIL/low TIL) | Gene symbol | Parametric p-value | fold change (high TIL/low TIL) |
|---|---|---|---|---|---|
| SNRPE | 0,018656 | 0,966889 | PCDH21 | 0,029458 | 1,05277 |
| GMFB | 0,018725 | 0,962138 | IQCA | 0,02948 | 0,963474 |
| ECGF1 | 0,019142 | 1,047679 | ZFP36L1 | 0,029489 | 0,970374 |
| NUCKS1 | 0,019547 | 0,970186 | HLA-DRB1 | 0,029529 | 1,031104 |
| WDR36 | 0,02033 | 0,962181 | GGTL4 | 0,029705 | 1,030813 |
| CARD9 | 0,020354 | 1,053982 | IDH3A | 0,029762 | 1,042238 |
| ARL13B | 0,020631 | 0,951318 | SPOCK1 | 0,029804 | 0,962544 |
| SFRP2 | 0,020799 | 0,926297 | TMEM38B | 0,029891 | 1,048449 |
| CCT6B | 0,020948 | 1,057199 | HYI | 0,029981 | 0,965 |
| C15orf48 | 0,021006 | 1,075849 | C8orf55 | 0,030142 | 1,028367 |
| NME3 | 0,02122 | 0,965483 | TMEM47 | 0,030284 | 0,966829 |
| SERPINA3 | 0,021544 | 1,04996 | PRSS22 | 0,03031 | 1,036198 |
| MYRIP | 0,021668 | 1,040777 | IRX1 | 0,030573 | 0,956758 |
| MLKL | 0,021702 | 1,046805 | GLT25D1 | 0,030707 | 0,966298 |
| GALNT5 | 0,021746 | 1,044584 | SRPX2 | 0,030712 | 0,95038 |
| SLC40A1 | 0,021875 | 1,048227 | COL11A1 | 0,03094 | 0,926402 |
| HEY2 | 0,021983 | 0,958874 | ZDHHC18 | 0,031512 | 1,030895 |
| MX1 | 0,022039 | 1,046448 | CCDC43 | 0,031636 | 0,969207 |
| ATP1A2 | 0,022163 | 0,954493 | RARRES3 | 0,031671 | 1,039329 |
| EGR1 | 0,022178 | 0,9673 | ANXA11 | 0,031961 | 0,966047 |
| ZNF91 | 0,022212 | 0,961726 | MGC24103 | 0,032138 | 0,962419 |
| HOXD10 | 0,022272 | 1,043444 | SGCB | 0,032169 | 0,961194 |
| TM9SF3 | 0,022464 | 0,963999 | FAM112B | 0,032185 | 1,057999 |
| HSD17B6 | 0,022521 | 0,948245 | DAD1 | 0,032559 | 0,971288 |
| JUND | 0,022585 | 0,974256 | TTYH2 | 0,032791 | 0,948983 |
| CYP4Z2P | 0,022618 | 1,05979 | SIRT6 | 0,032926 | 0,936267 |
| ACTN1 | 0,022886 | 0,970493 | NT5C2 | 0,033136 | 0,959484 |
| GBP5 | 0,023022 | 1,053155 | MAGEC1 | 0,033226 | 1,040437 |
| ARL8A | 0,023184 | 1,03448 | C1orf85 | 0,033301 | 0,969927 |
| GTF2H5 | 0,023662 | 0,964764 | HPS6 | 0,033599 | 0,971893 |
| RPS12 | 0,023997 | 0,984371 | ZFP42 | 0,033657 | 0,944134 |
| PARD3 | 0,02411 | 0,965383 | SERPINB5 | 0,033664 | 0,9477 |
| LOC339804 | 0,024268 | 1,045769 | PPP4R1 | 0,033733 | 1,065693 |
| C7orf23 | 0,024385 | 1,038642 | ITLN1 | 0,033816 | 1,035563 |
| COL9A3 | 0,02446 | 0,956419 | TEX264 | 0,034013 | 1,032305 |
| LOC388789 | 0,024749 | 0,969646 | C10orf71 | 0,034134 | 0,971296 |
| MAGEA2B | 0,024758 | 1,079554 | HMMR | 0,034151 | 1,06189 |
| TMEM144 | 0,025004 | 1,041673 | NDRG2 | 0,034473 | 0,972944 |
| TRIM47 | 0,025151 | 1,045587 | BARX1 | 0,034602 | 0,970175 |
| RUNX3 | 0,025661 | 1,047044 | GAS5 | 0,035271 | 0,968486 |
| IL8 | 0,025716 | 1,077473 | CXCR6 | 0,035424 | 1,039526 |
| OR4K1 | 0,025956 | 1,043061 | AMOTL2 | 0,035524 | 0,978737 |
| EBF1 | 0,026315 | 1,058574 | PLEKHF2 | 0,035701 | 1,04095 |
| HES6 | 0,026319 | 1,052962 | CARHSP1 | 0,035752 | 0,97425 |
| HMGN3 | 0,026475 | 0,971819 | STAT1 | 0,035871 | 1,036775 |
| SNHG5 | 0,026532 | 0,961322 | TNFRSF17 | 0,035997 | 1,070352 |
| C5orf26 | 0,026575 | 0,959577 | CCL18 | 0,036122 | 1,067768 |
| GZMA | 0,026672 | 1,054404 | KBTBD3 | 0,036206 | 1,06347 |
| GNL3L | 0,026721 | 1,036245 | DNMT3A | 0,036243 | 0,963485 |
| CTGF | 0,026844 | 0,956754 | ERVWE1 | 0,036425 | 1,052433 |
| TMSL4 | 0,027007 | 0,980662 | PRKCI | 0,036571 | 0,96471 |
| ALDH6A1 | 0,027111 | 0,96736 | RASGRP1 | 0,036672 | 1,044106 |
| GGT1 | 0,027765 | 0,936654 | NUDT17 | 0,036846 | 1,04814 |
| SCG3 | 0,027868 | 0,91115 | CEBPB | 0,037233 | 0,984643 |
| HOXA7 | 0,029009 | 0,961259 | GPAM | 0,037345 | 0,9631 |
| TXNDC10 | 0,029053 | 0,968704 | TSPAN13 | 0,037447 | 0,962836 |
| GSDMDC1 | 0,029442 | 1,041504 | MTMR9 | 0,037733 | 0,961098 |
| EIF3S10 | 0,029451 | 0,971334 | C11orf73 | 0,037812 | 0,971325 |

| Gene symbol | Parametric p-value | fold change (high TIL/low TIL) | Gene symbol | Parametric p-value | fold change (high TIL/low TIL) |
|---|---|---|---|---|---|
| CYB5A | 0,037822 | 0,97111 | PRELP | 0,044048 | 0,957666 |
| H1FOO | 0,038312 | 0,968405 | LTA4H | 0,044071 | 0,972399 |
| COL5A2 | 0,038324 | 0,950367 | ZBTB6 | 0,044176 | 1,036838 |
| LY75 | 0,038389 | 1,055312 | GPR31 | 0,044306 | 1,042828 |
| GUCY2F | 0,038394 | 1,064628 | CDK5R1 | 0,044415 | 0,969072 |
| TOMM7 | 0,038556 | 0,973502 | ADFP | 0,044437 | 0,954856 |
| FBN3 | 0,038733 | 0,942574 | ITGA8 | 0,044724 | 0,948782 |
| MARCKS | 0,038849 | 0,967259 | AGPAT2 | 0,044728 | 1,031039 |
| TF | 0,039044 | 0,95165 | VPS4B | 0,044807 | 0,96831 |
| SYDE1 | 0,039193 | 0,964285 | CREB3L4 | 0,044851 | 0,969375 |
| C1orf151 | 0,039363 | 1,037702 | GRM3 | 0,045086 | 1,037417 |
| CENPF | 0,039457 | 0,955777 | TAF13 | 0,045274 | 1,065998 |
| SEMA5A | 0,039527 | 0,957717 | C1orf78 | 0,045286 | 0,975181 |
| LDHB | 0,039572 | 0,972471 | SAMD9L | 0,04532 | 1,047637 |
| COX15 | 0,03971 | 0,963944 | CCDC80 | 0,045385 | 0,961262 |
| HLA-F | 0,039758 | 1,031955 | HSD11B1L | 0,045535 | 0,945436 |
| ZBTB26 | 0,039949 | 1,042478 | SP140 | 0,045633 | 1,060005 |
| FOSL1 | 0,040064 | 1,027777 | UCK2 | 0,045869 | 0,967043 |
| DHRS9 | 0,040106 | 1,047801 | TPM2 | 0,046075 | 0,968061 |
| FAM84A | 0,040423 | 0,969105 | PPP3CB | 0,046152 | 0,968526 |
| IRF4 | 0,040648 | 1,054497 | NEUROG3 | 0,046248 | 1,026144 |
| LAG3 | 0,040656 | 1,051308 | RPLP0P2 | 0,046301 | 0,982024 |
| RBBP7 | 0,040772 | 1,02828 | CDV3 | 0,046337 | 0,96992 |
| CAMK1G | 0,040862 | 1,059712 | C11orf71 | 0,046529 | 1,027314 |
| M6PRBP1 | 0,041344 | 0,971057 | NLRP7 | 0,047212 | 0,94897 |
| RPS13 | 0,041466 | 0,992434 | FGB | 0,047212 | 0,930181 |
| ATP6V1D | 0,04149 | 0,970117 | BZRPL1 | 0,047361 | 0,968624 |
| POLR2I | 0,04158 | 0,965969 | UQCRC1 | 0,047506 | 1,028321 |
| IL1R2 | 0,041643 | 1,031374 | SLC2A8 | 0,047528 | 1,037773 |
| AKAP12 | 0,041754 | 0,965955 | RPL23A | 0,047784 | 0,982168 |
| RKHD1 | 0,041812 | 0,971522 | ZNF307 | 0,047875 | 0,976348 |
| C17orf81 | 0,041819 | 0,95786 | OR5D14 | 0,048091 | 1,029101 |
| RNMTL1 | 0,041841 | 0,958277 | ERC1 | 0,048124 | 0,954965 |
| SYMPK | 0,041907 | 1,026278 | SNX9 | 0,048329 | 0,958665 |
| LOC116143 | 0,042157 | 1,036751 | CHRAC1 | 0,048371 | 1,039782 |
| PACRG | 0,042164 | 1,041308 | AK3 | 0,048466 | 1,037551 |
| HSD17B11 | 0,042209 | 0,964303 | IL32 | 0,0486 | 1,027019 |
| PUM2 | 0,042905 | 1,026572 | NAT1 | 0,048792 | 1,039456 |
| SUSD3 | 0,043025 | 1,041484 | TTLL12 | 0,048986 | 1,070338 |
| FNDC3B | 0,043043 | 0,967155 | HSP90AA1 | 0,049175 | 0,976061 |
| CNN1 | 0,04328 | 0,95379 | LRRFIP2 | 0,049324 | 1,027732 |
| TCF4 | 0,043448 | 0,965471 | ACAA2 | 0,04962 | 0,967112 |
| RRM2 | 0,043518 | 0,956725 | ZNF277P | 0,049645 | 0,967039 |
| B4GALT5 | 0,043699 | 0,966929 | CENPK | 0,049656 | 1,040088 |
| ZIC1 | 0,043757 | 0,941812 | RP9 | 0,049732 | 0,970948 |

# Chapter 6

## Antigen-specific active immunotherapy for ovarian cancer



Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief CJ, Nijman HW

*Submitted to Cochrane Database of Systematic Reviews*

## ABSTRACT

### Background

Despite advances in chemotherapy, prognosis of ovarian cancer remains poor. Antigen-specific active immunotherapy aims at the induction of tumour-antigen-specific anti-tumour immune responses as an alternative treatment for ovarian cancer.

### Objectives

To assess feasibility of antigen-specific active immunotherapy for ovarian cancer. Primary outcomes: clinical efficacy and antigen-specific immunogenicity. Secondary outcomes: carrier-specific immunogenicity and side-effects.

### Search strategy

A systematic search of the Cochrane Gynaecological Cancer Group Specialized Register, CENTRAL (issue 3, 2009), MEDLINE and EMBASE databases and clinicaltrials.gov was performed (1966 to July 2009). Hand searches were conducted of the proceedings of relevant annual meetings (1996 to July 2009).

### Selection criteria

Randomised controlled trials (RCTs), as well as non-RCTs that included patients with epithelial ovarian cancer, irrespective of stage of disease, and treated with antigen-specific active immunotherapy, irrespective of type of vaccine, antigen used, adjuvant used, route of vaccination, schedule, and reported clinical or immunological outcomes.

### Data collection and analysis

Data extraction was performed independently by two review authors. Risk of bias was evaluated with the Delphi-list (RCTs) or a selection of quality domains pivotal to the assessment of non-RCTs and deemed best applicable to the non-randomised non-controlled studies (non-RCTs).

### Results

Thirty-six studies were included. Response definitions showed substantial variation between trials, which makes comparison of trial results treacherous. Information on adverse events was frequently limited. Furthermore, reports of both RCTs and non-RCTs frequently lacked information necessary to assess risk of bias. Serious biases in these trials can thus not be ruled out.

The largest body of evidence is currently available for CA-125 targeted antibody therapy (15 studies: 1505 patients). Non-RCTs of this CA-125 targeted antibody therapy suggest increased survival in humoral and/or cellular responders. However, three large randomised placebo-controlled trials did not show any clinical benefit despite induction of immune responses in ~60% of patients.

Other small studies targeting many different tumour antigens showed promising immunological results. As these strategies have not yet been tested in RCTs, no reliable inferences about clinical efficacy can be made. Toxicity was generally limited. Given the promising immunological results and limited side effects, exploration of clinical efficacy in large well-designed RCTs may be worthwhile.

**Authors' conclusions**

We conclude that despite promising immunological responses no clinically effective antigen-specific active immunotherapy is yet available for ovarian cancer. Furthermore, the adoption of guidelines to ensure uniformity in trial conduct, response definitions and trial reporting is recommended to improve quality and comparability of immunotherapy trials.

## PLAIN LANGUAGE SUMMARY

Epithelial ovarian cancer is the most frequently diagnosed ovarian malignancy and the leading cause of death from gynaecological cancers. Standard therapy consists of surgery followed by chemotherapy. Although initial response rates are high, the majority of patients with advanced disease relapse. No curative treatment is available for recurrent disease. The observation that the presence of certain immune cells in tumours is associated with improved survival, suggests that stimulation of anti-tumour immune responses, i.e. immunotherapy, might be a useful approach to improve prognosis of ovarian cancer. In this survey, the feasibility of antigen-specific active immunotherapy is evaluated. Antigen-specific active immunotherapy aims at the induction of tumour-directed immune responses through the administration of a tumour-antigen, a molecule that is preferentially expressed by tumour cells and can induce immune responses. As immunotherapy is a novel treatment strategy early phase studies were also included. Information on clinical and immunological responses, and adverse events was collected.

Thirty-six studies in 1780 ovarian cancer patients were identified with a systematic search of study reports published between 1966 and 2009. The most frequently described strategy (1505 patients / 15 studies) was administration of antibodies targeting CA-125. Most of these primarily evaluated safety and immunological responses. Five studies described severe flu-like and gastro-intestinal symptoms in 7-30% of patients. Antibodies and immune cells recognising CA-125 were frequently detected, albeit response rates varied. Despite promising immunological responses, three large studies found equal survival rates for patients treated with placebo or CA-125 directed antibody. It thus seems that antibody therapy targeting CA-125 does not provide clinical benefit and should in its current form not be incorporated in standard treatment.

For strategies not relying on antibody administration, similar conclusions cannot be drawn based on the included studies as these have not yet been tested in large trials using a placebo control to evaluate clinical efficacy of treatment. These were generally small studies primarily investigating vaccine safety and immunogenicity. Overall, treatment was well-tolerated, with inflammatory side effects at injection site most frequently reported. Antibodies and immune cells were induced by most strategies studied, but their clinically activity still has to be evaluated in large trials.

Based on a lack of uniformity in included studies, we strongly advocate universal adoption of response definitions, guidelines for adverse events reporting, and directives for trial conduct and reporting.

## BACKGROUND

Epithelial ovarian cancer is the sixth most common cancer and the seventh cause of death from cancer in women worldwide (Parkin 2006). It is the second most common gynaecological cancer and the leading cause of death from gynaecological cancers in the Western world. Age standardized incidence rates range from 2.6 per 100,000 in Northern Africa to 13.3 per 100,000 in Northern Europe (Parkin 2006).

Stage of disease at presentation is the most important prognostic factor. Due to the asymptomatic course of disease, the majority of patients have extensive disease at presentation (stage III to IV according to FIGO classification (Benedet 2000)). Despite standard treatment, which consists of cytoreductive surgery and platinum-based chemotherapy, almost all patients with advanced stage disease at presentation will relapse, with a median progression-free survival (PFS) of only 18 months. When residual or recurrent disease manifests itself, resistance to chemotherapy often prohibits further curative therapy, resulting in a disease-specific five-year survival for patients with advanced stage ovarian disease of only 10 to 20% (Agarwal 2006; Thigpen 2000).

The immune system seems to play a role in ovarian cancer. This is reflected in the observation that in more than half of ovarian cancer patients, T-cells are present within tumour-islets (Raspollini 2005; Zhang 2003). Patients with advanced ovarian cancer, whose tumour is infiltrated by these T-cells, have a better clinical outcome compared to patients without these tumour-infiltrating T-cells (Dong 2006; Raspollini 2005; Zhang 2003). More specifically, higher numbers of cytotoxic T-cells, which can directly recognise and kill tumour cells, and increased ratios between cytotoxic T-cells ($CD8^+$) and helper T-cells ($CD4^+$) within the tumour epithelium are associated with improved survival (Sato 2005).

Immunotherapy is one of the novel therapeutic strategies under investigation in ovarian cancer. It aims at inducing or enhancing active immune responses directed towards the tumour to consolidate anti-tumour effects of standard therapy, delay and possibly prevent progression of disease. More specifically, antigen-specific active immunotherapy aims at activation of the adaptive immune system directed towards a specific target antigen through administration of a molecular defined antigen-specific vaccine to the patient. An antigen is a molecule, usually a protein or polysaccharide, which can stimulate an immune response. To obtain a tumour-specific immune response, immunotherapy exploits the differential expression of antigens between normal and tumour cells. Numerous antigens specific for tumour cells are known in ovarian cancer, e.g. sperm protein 17, MAGE-1, p53, Her-2/Neu. A major challenge concerning the safety of immunotherapy lies in the prevention of auto-immunity, i.e. induction of immune cells that preferentially recognise and kill tumour cells, but avoid destruction of normal body cells. From a theoretical point of view, other possible side

effects include allergic reactions to components of the vaccine and inflammatory reactions at the site of injection.

Several immunotherapeutic strategies are now being employed using different tumour antigens. These studies have, however, generally not yet evolved past phase I/II studies. To our knowledge, no systematic review of antigen-specific active immunotherapy in ovarian cancer has been carried out so far.

The immunogenicity and clinical efficacy of antigen-specific active immunotherapy in ovarian cancer is evaluated in this review. A systematic review about this topic is useful to ascertain the achievability of this treatment modality for ovarian cancer.

**Objectives**

The primary objective of this review was to assess the efficacy (i.e. clinical and/or immunological responses) of antigen-specific active immunotherapy for the treatment of ovarian cancer. The secondary objective was to establish which immunotherapeutic strategies combined with which tumour antigens provide the best immunological and clinical results.

## METHODS

### Criteria for considering studies for this review

#### *Types of studies*

We anticipated that there would be no randomised controlled clinical trials (RCTs) on this subject. Therefore we also included phase I, phase II non-randomised and non-controlled and if available phase III trials. We realise that results from non-randomised non-controlled trials cannot readily be extrapolated to the general population. Nevertheless, we feel that given the anticipated lack of RCTs, inclusion of these studies into this review was justifiable.

#### *Types of participants*

Women diagnosed with epithelial ovarian cancer, irrespective of stage of disease. However, as patient populations may differ substantially between different types of trials to be included in this review, for each trial we documented what type of patient was included into the trial (e.g. patients with end-stage disease or patients with residual disease).

Because we anticipated that there would not be many trials which included patients with ovarian cancer only, we also included immunotherapeutic studies in cancer patients that included at least two patients with ovarian cancer with the additional requirement that the results for these individual patients would have to be separately identifiable (from the trial publication or communication with the author). From these studies, only data on the patients with ovarian cancer was extracted for the review. We are fully aware of the cautions necessary when drawing conclusions based on studies with such small numbers, but felt that given the anticipated lack of large RCTs, inclusion of these studies into this review was justifiable.



#### *Types of interventions*

Antigen-specific active immunotherapy is defined as therapy which aims at inducing an adaptive immune response directed towards the tumour by means of administration of a specific well-defined tumour antigen. We compared interventions with each other based on the above-mentioned characteristics.

We included all interventions that aimed at antigen-specific active immunotherapy irrespective of type of vaccine, antigen used, adjuvant used, route of vaccination, vaccination schedule.

**Types of outcome measures**

***Primary outcomes***

Clinical efficacy

To assess clinical efficacy we evaluated:

1. Tumour responses to immunotherapy (complete / partial response, stable / progressive disease), as measured by:

- CA-125 levels according to or transposable to GCIG criteria (Rustin 2004)
- Tumour response according to WHO criteria or RECIST criteria (WHO 1979; Therasse 2000)

2. If available we evaluated responses to post-immunotherapy treatment, as there are indications that patients treated with chemotherapy after immunotherapy have increased survival as opposed to patients who did not receive immunotherapy (Antonia 2006).

3. If available, survival differences based on treatment with immunotherapy.

Antigen-specific immunogenicity

We recorded the number of observed antigen-specific humoral and cellular responses. When possible, we separately reported responses of cytotoxic ($CD8^+$) T-lymphocytes and/or helper ($CD4^+$) T-lymphocytes.



***Secondary outcomes***

Carrier-specific immunogenicity

As certain immunotherapeutic strategies rely on the use of carriers that may be the subject of an immune response besides the intended antigen-specific immune response, we recorded information on the induction of carrier-specific immune responses when appropriate.

Adverse events

To obtain an impression of the toxicity of antigen-specific immunotherapy, we extracted information on adverse events observed and reported in the different studies. Adverse events were categorised as local adverse events at the site of immunisation or systemic adverse events (all other reported adverse events). Systemic adverse events were subdivided into autoimmunity, allergic reactions and other adverse events occurring after immunisation.

**Search methods for identification of studies**

Cochrane Gynaecological Cancer Group Specialized Register, Cochrane Central Register of Controlled Trials (CENTRAL - issue 3, 2009) as well as the prospective trial register www.clinicaltrials.gov were searched. Furthermore, we searched MEDLINE (1966 to July 2009) and EMBASE (1974 to July 2009) according to the search strategies listed in Appendix 1 and Appendix 2.

Hand searching was undertaken of abstracts in the proceedings of annual meetings of Society of Gynecologic Oncologists, the American Association for Cancer Research and the International Society for Biological Therapy of Cancer (1996 to July 2009).

The bibliography of each primary reference and of recent reviews of immunotherapy for ovarian cancer was checked for additional trial publications. In addition we wrote to specialists involved in research regarding immunotherapy for ovarian cancer for information about the results of unpublished or ongoing studies. Relevant data were included in this review.

There were no language restrictions other than those inherent to the databases surveyed.

**Data collection and analysis**

***Selection of studies***

All titles and abstracts retrieved by electronic searching were downloaded to Reference Manager, duplicates were removed and the remaining references were examined by two review authors (HWN and NL) independently. Those studies which clearly did not meet the inclusion criteria were excluded and copies of the full text of potentially relevant references were obtained. The eligibility of retrieved papers was assessed independently by two review authors (HWN and NL). Differences between review authors were resolved by discussion or by appeal to a third review author if necessary (TD). Reasons for exclusion were documented.



***Data extraction and management***

For included studies, data on characteristics of patients and interventions, study quality and endpoints were extracted independently by two review authors (HWN and NL) onto a data extraction form specially developed for the review.

Where data on clinical efficacy and antigen-specific immunogenicity were missing from reports, we attempted to contact the authors to obtain the missing information. Results were checked for accuracy by a third review author (WH or TD).

***Assessment of risk of bias in included studies***

Risk of bias in RCTs complying with our selection criteria was assessed according to the Delphi-list (Verhagen 1998). Studies were evaluated based on randomisation, concealment of treatment allocation, blinding of patient, caregiver and outcome assessor, baseline similarity of groups, intention-to-treat (ITT) analysis, specification of eligibility criteria, and presentation of point estimates and measures of variability for the primary outcome measures.

No standard tools to evaluate validity are available for non-RCTs. Instead, for these trials we evaluated the risk of bias using the following four domains (Table 1):

- sample definition and selection
  - clear definition of inclusion / exclusion criteria
  - representative selection
  - adequate description of baseline characteristics
- interventions:
  - clear specification
  - concurrent / concomitant treatment
- outcomes:
  - specifications of outcome measures
  - relevance of outcome measures
  - reporting of outcome measures
- statistical analysis:
  - adequate rationale for number of patients included
  - adequate description withdrawal / exclusion during the study
  - adequate presentation of results.

These domains were selected as representative for and applicable to non-randomised non-controlled studies from a list of 12 quality domains and items deemed to be pivotal to the assessment of non-RCTs (Deeks 2003).
Risk of bias assessment was carried out by two review authors (HWN & NL). Discrepancies between review authors were solved by discussion; if necessary a third author (WH or TD) was consulted.



***Data synthesis***

This review provides a narrative analysis, because included trials are highly heterogeneous regarding intervention and outcome measures. Furthermore data in publications were often presented with insufficient details (lack of standard deviations (SDs) or only some of the multiple outcomes presented), and additional information from report authors was difficult to obtain. Therefore we felt that quantitative meta-analysis and calculation of effect size estimates would neither be meaningful nor appropriate in this review. We limited analysis to a structured summary and discussion of available studies and findings.

**Table 1** Trial Report Quality Assessment for non-randomised, non-controlled trials

| Item | Question | Evaluation |
|---|---|---|
| 1. | **Sample Definition and Selection** | |
| a. | Are the in- and exclusion criteria clearly defined? | Yes No Unk |
| b. | Is the study population a representative selection of the true population? | Yes No Unk |
| c. | Are baseline characteristics adequately described? | Yes No Unk |
| 2. | **Interventions** | |
| a. | Are the interventions clearly described (type of vaccine, antigen, adjuvant, route of vaccination and vaccination schedule)? | Yes No Unk |
| b. | Did patients receive concurrent / concomitant treatment with immunomodulatory effects? | Yes No Unk |
| 3. | **Outcomes** | |
| a. | Are the selected outcome measures clearly specified? | Yes No Unk |
| b. | Are the outcome measures relevant? | Yes No Unk |
| c. | Are the outcome measures clearly reported? | Yes No Unk |
| 4. | **Statistical Analysis** | |
| a. | Is there an adequate rationale for the number of patients included? | Yes No Unk |
| b. | Is there an adequate description of withdrawal / exclusion of patients during the study? | Yes No Unk |
| c. | Is the presentation of the results adequate? | Yes No Unk |

Unk - unknown



**Table 2** Assessment of randomised controlled trial quality according to Delphi Checklist

| Study | | N | randomisation | concealed treatment allocation | groups similar at baseline | eligibility criteria specified | outcome assessor blinded | caregiver blinded | patient blinded | point estimates and measures of variability | intention-to-treat analysis | additional comment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | yes | unk | yes | unk | yes | yes | yes | no | unk | abstract |
| Berek | 2004 | 145 | yes | unk | yes | yes | yes | unk | unk | yes | yes | full text |
| Berek | 2009 | 371 | yes | yes | yes | yes | yes | yes | yes | yes | unk | full text |
| Braly | 2009 | 40 | yes | unk | no | no | unk | no | no | yes | unk | full text |
| Chu | 2008 | 14 | yes | unk | unk | unk | unk | no | no | yes | unk | abstract |
| Freedman | 1998 | 30 | yes | unk | unk | unk | yes | unk | yes | yes | unk | abstract |
| Herrin | 2007 | 21 | yes | unk | unk | unk | unk | no | no | no | unk | abstract |
| Method | 2002 | 102 | yes | unk | yes | unk | unk | no | no | no | unk | abstract |
| Sabbatini | 2006 | 42 | yes | unk | unk | yes | unk | no | no | yes | unk | full text |

Unk - unknown

**Table 3** Assessment of trial report quality of non-randomised (un)controlled trials

| | | N | Clear definition of in-/ exclusion criteria | Representative of true population | Baseline characteristics adequately described | Interventions clearly described | Concomitant / concurrent immunomodulatory treatment | Outcome measures clearly specified | Outcome measures relevant | Outcome measures clearly defined | Adequate rationale for number of patients | Adequate description of exclusion / withdrawal | Adequate presentation of results |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Brossart | 2008 | 3 | yes | unk | no | yes | unk | yes | yes | yes | no | no | no |
| Chianese - Bullock | 2008 | 9 | yes | no | yes | yes | unk | yes | yes | yes | no | yes | no |
| Diefenbach | 2008 | 9 | yes | no | yes | yes | no | yes | yes | yes | no | yes | yes |
| Ehlen | 2005 | 13 | yes | yes | yes | yes | unk | yes | yes | yes | no | yes | yes |
| Gordon | 2004 | 20 | yes | yes | yes | yes | yes | yes | yes | yes | no | no | yes |
| Gulley | 2008 | 3 | yes | unk | no | yes | unk | yes | yes | yes | no | yes | no |
| Herrin | 2007 | 20 | yes | yes | yes | yes | yes | yes | yes | yes | no | no | yes |
| Leffers | 2009a | 20 | yes | unk | yes | yes | no | yes | yes | yes | yes | yes | yes |
| Ma | 2002 | 4 | no | unk | no | no | unk | no | no | no | no | no | no |
| MacLean | 1992 | 10 | no | unk | no | yes | yes | yes | yes | yes | no | no | yes |
| MacLean | 1996 | 34 | yes | unk | no | yes | yes | no | yes | no | no | yes | no |
| Möbus | 2003 | 44 | yes | yes | yes | yes | yes | no | yes | yes | no | no | yes |
| Mohebtash | 2009 | 17 | no | unk | no | yes | unk | no | yes | no | no | no | no |
| Nicholson | 2004 | 26 | yes | unk | no | yes | unk | yes | yes | yes | no | yes | yes |
| Nishikawa | 2006 | 4 | no | unk | no | no | unk | yes | yes | yes | no | no | no |
| Noujaim | 2001 | 184 | yes | yes | yes | no | unk | yes | yes | yes | no | no | yes |
| Odunsi | 2007 | 18 | no | no | yes | yes | unk | no | yes | yes | no | unk | yes |
| Odunsi | 2007a | 19 | no | unk | no | yes | unk | no | yes | no | no | no | no |
| Pfisterer | 2006 | 36 | yes | unk | no | yes | unk | yes | yes | yes | no | yes | yes |
| Reinartz | 2004 | 119 | yes | unk | no | yes | no | yes | yes | yes | no | no | yes |
| Sabbatini | 2000 | 25 | yes | yes | yes | yes | unk | no | yes | yes | no | yes | yes |

*Table 3 - continued*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sabbatini | 2007 | 11 | yes | unk | yes | yes | unk | yes | yes | yes | yes | yes | no |
| Sandmaier | 1999 | 7 | yes | unk | no | yes | no | no | yes | yes | no | yes | yes |
| Schultes | 1998 | 75 | no | unk | no | yes | unk | no | yes | yes | no | no | yes |
| Ströhlein | 2009 | 2 | yes | no | no | yes | unk | yes | yes | yes | no | yes | yes |
| Tsuda | 2004 | 5 | yes | no | no | yes | no | yes | yes | no | no | yes | no |
| van Zanten-Przybysz | 2002 | 5 | yes | no | yes | yes | unk | yes | yes | yes | no | yes | yes |
| Wagner | 1993 | 58 | no | unk | no | yes | unk | no | yes | no | no | no | no |

Unk - unknown

## RESULTS

### Description of studies

#### *Results of the search*

From the electronic searches of MEDLINE and EMBASE, 56 abstracts were selected as potentially compliant with the selection criteria and full texts were retrieved. Evaluation of the retrieved full texts resulted in the exclusion of 26 papers because of various reasons (see Excluded studies). In addition to the 30 selected full texts, another 14 abstracts were identified by hand searching the proceedings of the periodic meetings specified in the methods section. Authors were contacted for manuscripts, but no full texts were obtained for these abstracts. Together the 44 selected full texts and meeting abstracts described a total of 35 studies. Search of the prospective trial register www.clinicaltrials.gov (search terms: ovarian cancer + vaccine or ovarian cancer + immunotherapy) resulted in identification of an additional 26 studies. For only four of these a full text or meeting abstract could be retrieved and only one study complied with our inclusion criteria (excluded: Marshall 2005; Morse 2003; Salazar 2006; included: Sabbatini 2007). The remaining trials were either ongoing (n=15) or completed but not yet published (n=6; NCT00019084; NCT00019916; NCT00034138; NCT00034372; NCT00381173; NCT00585845). Search of CENTRAL (version 3, 2009) did not result in identification of any additional trials. Thus in total, 36 studies were included in this review. Generally, the most recent peer-reviewed publication was selected as the primary reference.



#### *Included studies*

The 36 studies included in this review were all published in English.

Design

As expected the majority of studies were uncontrolled phase I or II trials (27 out of 36). Only three studies were randomised placebo controlled studies (Berek 2001; Berek 2004; Berek 2009). Randomized allocation of patients to different regimens was used in six studies (Braly 2009; Chu 2008; Freedman 1998; Herrin 2007; Method 2002; Sabbatini 2006). In four studies the immunogenicity of a previously applied immuno-scintigraphic agent was retrospectively studied (Möbus 2003; Noujaim 2001; Schultes 1998; Wagner 1993).

Sample sizes

The median number of patients treated per study was 20 (range 2-371). Nine studies included less than 10 patients. Six studies also included patients with other types of cancer (Brossart 2000; Gulley 2008; Mohebtash 2009; Sandmaier 1999; Ströhlein 2009; Tsuda 2004). A sample size calculation or rationale was provided for six studies only (Berek 2004; Berek 2009; Braly 2009; Leffers 2009a; Sabbatini 2006; Sabbatini 2007).

Participants

As was expected, the disease status at study entry varied largely between trials. Patients with evidence of residual or recurrent disease after treatment were most frequently included (13 out of 36) (Freedman 1998; Gulley 2008; Leffers 2009a; Ma 2002; Method 2002; Mohebtash 2009; Nishikawa 2006; Pfisterer 2006; Reinartz 2004; Sandmaier 1999; Schultes 1998; Ströhlein 2009; Wagner 1993). Four trials included patients with and without evidence of disease after prior therapy (Braly 2009; Chianese-Bullock 2008; Tsuda 2004; Odunsi 2007). Eight trials included patients with complete response to therapy for primary or recurrent disease (Berek 2001; Berek 2004; Berek 2009; Chu 2008; Diefenbach 2008; Odunsi 2007a; Sabbatini 2000; Sabbatini 2007). In addition, one trial also included patients with minimal residual disease after primary therapy (Sabbatini 2006). In one study only was treatment administered together with adjuvant chemotherapy after primary cytoreductive surgery (Braly 2009). For the remaining nine trials disease status at entry was not reported.

Interventions

The majority of studies described antibody therapy (18 out of 36), usually targeting CA-125 (15 out of 18). Most studies included only one target antigen in the vaccine, but in six studies multiple antigens were simultaneously targeted (Chianese-Bullock 2008; Chu 2008; Gulley 2008; Mohebtash 2009; Sabbatini 2007; Tsuda 2004). Antibodies were usually administered intravenously (11 out of 18). For other vaccine types, subcutaneous injections were most common (13 out of 18).

Concurrent treatment with immunomodulatory drugs was not allowed in 8 out of 36 studies. In an additional 11 studies, concomitant immunomodulatory agents were not part of the studied intervention, but no explicit statements were made about prohibition of such drugs in the protocol. In ten studies immunomodulatory drugs were part of the protocol (i.e. carboplatin-paclitaxel, cyclophosphamide, IL-2 +/- GM-CSF, or diphenhydramine) and one study allowed interruption of immunotherapy by chemotherapy for progressive disease (Reinartz 2004). Furthermore, two retrospective studies explicitly mentioned concurrent chemotherapy (Möbus 2003; Wagner 1993).

Outcomes

Information on immunological responses, clinical responses, survival and adverse events was available for 34, 21, 25 and 28 studies respectively.

***Excluded studies***

Frequent reasons for exclusion were inclusion of too few ovarian cancer patients and the impossibility to distinguish results of ovarian cancer patients from other patients.

**Table 4** Evaluation of clinical responses to immunotherapy

| Study | | n | analyzed | method | CA-125 | result | tumour | result | overall conclusion |
|---|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | no | | | | | | |
| Berek | 2004 | 145 | no | | | | | | |
| Berek | 2009 | 371 | no | | | | | | |
| Braly | 2009 | 40 | yes | unk | | | | | cCR 15x / 17x |
| Brossart | 2008 | 3 | yes | unk | | | | | 2x SD, 1x PD |
| Chianese-Bullock | 2008 | 9 | yes | both | unk | | unk | | 1x NED, 8x PD |
| Chu | 2008 | 14 | yes | unk | | | | | 3x PD, 7x NED |
| Diefenbach | 2008 | 9 | yes | both | unk | | unk | | n.r. |
| Ehlen | 2005 | 13 | yes | both | decrease >15% (↓); <15% change (=) stable; >15% increase (↑) | 4x ↓, 1x =, 6x ↑ | unk | | 3x SD, 10x PD |
| Freedman | 1998 | 30 | yes | unk | | | | | 18x SD, 10x PD |
| Gordon | 2004 | 20 | yes | both | unk | 6x ↓ | unk | | 2x NED, 2x CR, 1x PR, 1x SD, 9x PD |
| Gulley | 2008 | 3 | yes | both | unk | | unk | | n.r. |
| Herrin | 2007 | 21 | yes | unk | | | | | 8x PD, 3x NED |
| Leffers | 2009a | 20 | yes | both | GCIG | n.r. | RECIST | n.r. | 2xSD, 18xPD |
| Ma | 2002 | 4 | no | | | | | | |
| MacLean | 1992 | 10 | yes | unk | | | | | 3x SD, 7x PD |
| MacLean | 1996 | 34 | no | | | | | | |
| Method | 2002 | 102 | yes | unk | | | | | n.r. |
| Möbus | 2003 | 44 | no | | | | | | |
| Mohebtash | 2009 | 17 | no | | | | | | |
| Nicholson | 2004 | 26 | yes | CA-125 | unk | | | | 21x PD, 1x SD, 1x l.f.u., 3x unk |
| Nishikawa | 2006 | 4 | no | | | | | | |
| Noujaim | 2001 | 184 | no | | | | | | |
| Odunsi | 2007 | 18 | yes | | | | unk | | 1x CR, 17x unk |
| Odunsi | 2007a | 19 | no | | | | | | |
| Pfisterer | 2006 | 36 | no | | | | | | |
| Reinartz | 2004 | 119 | yes | tumour | | | WHO | | n.r. |

**Table 4,** *continued*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sabbatini | 2000 | 25 | no | | | | | | |
| Sabbatini | 2006 | 42 | yes | both | unk | | unk | | 12x SD, 21x PD, 9x withdrawal (6x PD) |
| Sabbatini | 2007 | 11 | no | | | | | | |
| Sandmaier | 1999 | 7 | no | | | | | | |
| Schultes | 1998 | 75 | no | | | | | | |
| Ströhlein | 2009 | 2 | yes | both | unk | | unk | | 1xPD, 1xPR or SD |
| Tsuda | 2004 | 5 | yes | both | unk | | WHO | | 4x PD, 1x SD |
| van Zanten-Przybysz | 2002 | 5 | yes | both | unk | 1x ↓, 1x =, 1x ↑, 2x unk | unk | 1x NED, 1x SD, 2x PD, 1x unk | 3xPD, 2xSD |
| Wagner | 1993 | 58 | yes | CA-125 | unk | | | | n.r. |

Unk – unknown; l.f.u. – lost in follow-up; cCR – complete clinical remission; CR – complete response; PR – partial response; SD – stable disease; PD – progressive disease; NED – no evidence of disease; n.r. – not reported

**Risk of bias in included studies**

The assessment of risk of bias by means of the Delphi list was hindered by the fact that for five of these nine trials only meeting abstracts were available (Table 2). The four trials for which full texts were retrieved also did not report on some items of the Delphi list. Overall this resulted a median of four unreported items (range one to five) per study. With view to this substantial lack of information, it is too treacherous to make any statement about biases in and validity of randomised trials.

An overview of the assessment of trial report quality and risk of biases of the non-randomised trials is provided in Table 3. Important observations from this table are the lack of clearly defined in-/exclusion criteria in 8 out of 27 studies combined with the serious underreporting of baseline characteristics (16 out of 27 studies) which makes it impossible to evaluate whether the study populations were representative of the true population. Although the investigational interventions were well described in the majority of trials (24 out of 27), information on the allowance or application of concomitant immunomodulatory treatment was frequently absent (18 out of 27). Albeit a clear description of outcome measures was available for 17 studies, an adequate calculation of sample size based on a clearly defined primary outcome measure was available for only two studies. Furthermore, the applied checklist shows that the justification of withdrawals and exclusions during the trial as well as the presentation of trial results are items that require serious attention in the reports of these non-randomised trials.



Based on the above, the risk of bias in the studies included in this systematic review cannot be neglected. Especially selection bias (selection of a treatment population not comparable to control group or true population), attrition bias (inadequate reporting of withdrawal and exclusions during the trial resulting in possible over- or underestimation of effect) and selective reporting bias are likely to affect the studies included in this review. The effects of interventions described below must therefore be interpreted with prudence.

**Effects of interventions**

***Primary outcomes***

Clinical efficacy: tumor responses

Clinical responses to therapy were evaluated in 21 studies (Table 4). In the reports on these studies, criteria for evaluation and/or explicit description of tumour responses per patient as well as time point at which the evaluation took place were however frequently not available. For studies that did mention evaluation of tumour responses, response outcomes were based on either CA-125 levels combined with tumour imaging (Chianese-Bullock 2008; Diefenbach 2008; Ehlen 2005; Gordon 2004; Gulley 2008; Leffers 2009a; Sabbatini 2006; Ströhlein 2009; Tsuda 2004; van Zanten-Przybysz 2002), CA-125 alone (Nicholson 2004; Wagner 1993) or imaging alone (Odunsi 2007; Reinartz 2004). Only two studies explicitly mentioned evaluation of imaging according to the internationally accepted WHO or

**Table 5** Definitions and results of survival analysis in antigen-specific monoclonal antibody studies

| Study | | analyzed | definition | results |
|---|---|---|---|---|
| Berek | 2001 | yes | time to relapse | NS: median TTR placebo 11.3, robust HAMA 16.4, and robust Ab2 18.9 months |
| Berek | 2004 | yes | time to relapse / overall survival | NS: TTR oregovomab – 24.0 vs. placebo – 10.8 months (HR 0.543, 95%CI 0.287-1.025); OS 57.5 oregovomab vs. 48.6 placebo (HR 0.72, 95%C.I. 0.41-1.25) |
| Berek | 2009 | yes | time to relapse (randomisation to relapse) | NS: median TTR oregovobomab 10.3 months vs. placebo 12.9 months |
| Braly | 2009 | yes | Progression-free survival | NS: median PFS simultaneous administration 17.9 months vs. delayed administration 16.1 months |
| Ehlen | 2005 | yes | time to progression / survival (first dose to death) | TTP median 8.4 weeks (range 2-61 weeks); survival 37 weeks (range 11-110) |
| Gordon | 2004 | yes | time to progression / survival (first dose to death) | TTP median 11 weeks (T-cells responders vs. non-responders P<0.0001 HR 0.150, 95%CI 0.006-0.168); survival median 70.4 weeks (T-cell responders vs. non-responders P<0.002 HR 0.157, 95%CI 0.009-0.347) |
| Ma | 2002 | no | | |
| Method | 2002 | no | | |
| Möbus | 2003 | yes | survival (first dose to death) / overall survival (diagnosis to death) | survival median 16.8 months 95% CI 10.3-22.6 (Ab3 responders vs. non-responders 18.2 vs. 13.1, p=0.0896; HAMA responders vs. non-responders 22.6 months vs. 7.6 months, p=0.0016); overall survival median 34.4 months |
| Nicholson | 2004 | no | | |
| Noujaim | 2001 | yes | survival (first dose to death) | median survival & 3-year survival: Ab3 responders vs. non-responders 22.9 vs. 13.5 months, p=0.0089 – 38% vs. 8%; T-cell responders vs. non-responders (n=16) >84 vs. 13.2 months, p=0.0202 – 75% vs. 0%. |
| Pfisterer | 2006 | no | | |
| Reinartz | 2004 | yes | survival (first dose to death) | median survival 19.4 months, Ab3 responders vs. non-responders: 23.4 vs. 4.9 months, p<0.0001 |
| Sabbatini | 2006 | yes | time to progression | TTP: 4 months (95%CI 3-5 months) |
| Schultes | 1998 | yes | overall survival (diagnosis to death) | median OS: robust Ab3 responders vs. non-robust responders 49 vs. 38 months , p=0.0029; Ab2 robust vs. non-robust responders 30.0 vs. 44.0 months, p=0.0475 |
| Ströhlein | 2009 | yes | overall survival | not described separately for ovarian cancer patients |
| van Zanten-Przybysz | 2002 | yes | survival (first dose to death) | median survival 22.0 months |
| Wagner | 1993 | yes | not described | survival robust Ab2 vs. non-robust Ab2 responders NS |

TTR – time to relapse; TTP – time to progression; OS – overall survival; PFS – progression-free survival; HR – hazard ratio; CI – confidence interval; HAMA – human anti-mouse antibody; Ab2 – anti-idiotypic antibody; Ab3 – anti-anti-idiotypic antibody

**Table 6** Definitions and results of survival analysis in other antigen-specific immunotherapy trials

| Study | | analyzed | definition | results |
|---|---|---|---|---|
| Brossart | 2008 | no | | |
| Chianese-Bullock | 2008 | no | | |
| Chu | 2008 | no | | |
| Diefenbach | 2008 | yes | time to progression (last chemo to relapse) | median TTR 13.0 months (95%CI 11.2 – not reached) |
| Freedman | 1998 | yes | progression-free interval; survival | median PFI 4 months (95%CI 1.9-7.6); median survival 13.3. months (95%CI 1.5 – 30.8) |
| Gulley | 2008 | yes | progression-free survival; overall survival | PFS: 9, 18, 19$^{+}$ months; OS: 6, 19$^{+}$, 21 months |
| Herrin | 2007 | yes | progression-free survival; overall survival | mean PFS 5 months; mean OS SQ vs. IV 70.4 vs. 72.9 months |
| Leffers | 2009a | no | | |
| MacLean | 1996 | yes | survival (trial entry to death) | median survival 12.7 months |
| MacLean | 1992 | no | | |
| Mohebtash | 2009 | yes | time to progression | median TTP 2 months (range 1-36) |
| Nishikawa | 2006 | no | | |
| Odunsi | 2007 | yes | time to progression (first vaccination to relapse) | median TTP 19.0 months (95% CI 9.0 – not reached) |
| Odunsi | 2007a | yes | disease-free survival | median DFS 19.9 months |
| Sabbatini | 2000 | yes | time to progression (trial entry to relapse) | median TTP 6 months (range 2-17) |
| Sabbatini | 2007 | Yes | time to progression (first vaccination to relapse) | median TTP 4.2 months (95% CI 2.7-8.5) |
| Sandmaier | 1999 | no | | |
| Tsuda | 2004 | no | | |

TTR – time to relapse; PFI – progression-free interval; PFS – progression-free survival; DFS – disease-free survival; CI – confidence interval; SQ – subcutaneous; IV – intravenous

RECIST criteria (Leffers 2009a; Reinartz 2004; Tsuda 2004) and only two studies evaluated CA-125 levels according to GCIG criteria or described CA-125 levels in such a way that evaluation according to these criteria was possible for at least some patients (Leffers 2009a; van Zanten-Przybysz 2002). Strikingly, five studies stated that evaluation of tumour responses was performed, but results could not be found in the publications (Diefenbach 2008; Gulley 2008; Method 2002; Reinartz 2004; Wagner 1993). Complete or partial tumour responses in patients with evidence of disease at study entry were reported by only two studies (Gordon 2004; Odunsi 2007) in a small fraction of patients (3 out of 15 and 1 out of 18 respectively). These results however need to be interpreted with caution as criteria for response evaluation were not defined.

Clinical efficacy: responses to 'secondary' treatment after immunotherapy

Although studies generally have a period of follow-up to obtain information on survival, in the majority of studies no report is given of subsequent treatment with and response to secondary chemotherapy. Seven studies mention that patients were treated with chemotherapy after immunotherapy (Berek 2004; Gordon 2004; Möbus 2003; Odunsi 2007; Reinartz 2004; Ströhlein 2009; van Zanten-Przybysz 2002), but only two studies, both investigating a monoclonal antibody targeting CA-125, report response to secondary chemotherapy in relation to immunological responses to immunotherapy (Gordon 2004; Reinartz 2004). In a preliminary report clinical responses of 28 out of 42 patients treated with chemotherapy for clinically relevant progression during or after antibody therapy were reported in conjunction with the induction of human-anti-mouse and anti-anti-idiotype antibodies. Although both patients with a complete response had strong humoral responses, similar or stronger antibody responses were also observed for patients with stable or progressive disease (Reinartz 2004). In the other study, shortly after monotherapy with a monoclonal antibody, 13 out of 20 patients received chemotherapy combined with the monoclonal antibody. In this study, clinical responses to chemo-immunotherapy, were only observed in patients with cellular responses to CA-125 and/or autologous tumour (Gordon 2004).



Clinical efficacy: survival

Definitions of survival used in the different studies greatly varied (Table 5 and Table 6). Furthermore, reliable statements about survival (dis)advantages can only be made based on randomised controlled trials. Only three studies were designed to primarily evaluate survival, however no statistically significant differences in time to relapse and/or overall survival were found between patients treated with a monoclonal antibody or placebo (Berek 2001; Berek 2004; Berek 2009). Many non-randomised studies have also evaluated survival, frequently by comparing survival of patients with robust immunological responses to patients with no or weak immunological responses to treatment (Table 5 and Table 6). These results should however be interpreted with great caution as shorter survival in non-responders could

merely be a reflection of the general condition of these patient and well-known clinical and pathological prognostic parameters.

Antigen-specific immunogenicity

*Humoral responses*

Monoclonal antibodies may induce anti-idiotype antibodies (Ab2), directed primarily against the administered monoclonal antibody, as well as anti-anti-idiotype antibodies (Ab3) directed towards the target antigen. Anti-idiotype and anti-anti-idiotype antibodies were evaluated in 10 and 9 out of 18 studies respectively (Table 7 and Table 8). Response percentages greatly varied (Ab2: 3 to 100%, Ab3: 0 to 100%).

Eight studies (10 out of 18) of other vaccine types evaluated the induction of antigen-specific antibodies by ELISA, however only two studies clearly defined when an antibody titre or concentration was considered positive (Table 9) (Diefenbach 2008; Sandmaier 1999). Large differences in percentages of patients with measurable antigen-specific antibodies (IgG: 0 to 96%) existed. Possible explanations for these broad ranges are differences in 1) response definition, 2) number of treatment cycles after which humoral responses were measured, and 3) targeted antigen.

*Cellular responses*

The induction of T-cells against the target antigen was investigated in 11 out of 18 monoclonal antibody studies (Table 10). The presence of antigen-specific T-cells was evaluated by commonly used tests, such as IFN-γ ELISPOT (Ehlen 2005; Gordon 2004; Method 2002; Sabbatini 2006), proliferation assay (Ma 2002; Noujaim 2001; van Zanten-Przybysz 2002), cytokine profiling (Noujaim 2001; Pfisterer 2006) and IFN-γ secretion assay (Ströhlein 2009). One study used the leukocyte migration inhibition assay (Wagner 1993), which nowadays is rarely used. As described above for humoral responses, response definitions were frequently lacking or inadequate. Nevertheless, cellular immunity against CA-125 was reported for 21 to 80% of patients. Antibody treatment targeting the membrane folate receptor however did not induce cellular responses (van Zanten-Przybysz 2002). Recognition of autologous tumour cells by induced T-cells was determined in two trials only, with positive responses in 5/8 and 1/2 patients respectively (Gordon 2004; Ströhlein 2009).

Antigen-specific cellular immune responses were evaluated for 12 out of 18 studies using other vaccine types (Table 11). The most frequently used assay was the IFN-γ ELISPOT assay, which was sometimes used to separately analyse helper T-lymphocytes and/or cytotoxic T-lymphocytes. Again response definitions for positive and/or vaccine-induced responses were frequently absent or unclear (8 out of 18). In four studies NY-ESO-1 specific T-cells were induced, with percentages of patients with NY-ESO-1-specific cytotoxic T-cells ranging from 33 to 67% (Diefenbach 2008; Odunsi 2007; Odunsi 2007a). After treatment with a vaccine targeting p53, p53-specific T-cells were observed in approximately 70% of patients, irrespective of whether short peptides or peptide-pulsed dendritic cells were used (Herrin 2007).

Lastly, studies targeting multiple antigens demonstrated antigen-specific cellular immunity with varying immunogenicity of the different antigens targeted (Brossart 2000; Chianese-Bullock 2008; Chu 2008; Tsuda 2004).

### *Secondary outcomes*

**Carrier-specific immunogenicity**

The majority of studies using a monoclonal antibody (17 out of 18) used a murine antibody and one study used a chimeric antibody construct (van Zanten-Przybysz 2002). Next to antigen-specific immunity, the induction of human-anti-mouse antibodies (HAMA) using HAMA-specific ELISA assays was assessed in 13 studies (Table 12). HAMA were present in 4-97% of patients immunized (Berek 2004; Braly 2009; Ehlen 2005; Gordon 2004; Method 2002; Möbus 2003; Pfisterer 2006; Reinartz 2004; Sabbatini 2006; Schultes 1998). It seems that the large variation between studies cannot be attributed to differences in dosage, but is best ascribed to different definitions of a HAMA response i.e. some studies only report robust responses, whereas others report all responses above a certain threshold. Furthermore, the point in time at which HAMA titers were measured is of importance as responses increase in frequency and strength with repeated administrations of the antibody (Gordon 2004; Method 2002; Möbus 2003).

Although six studies investigated synthetic carbohydrate antigens conjugated to the keyhole limpet haemocyanin (KLH) carrier protein (MacLean 1992; MacLean 1996; Sabbatini 2000; Sandmaier 1999; Sabbatini 2007), only one study reported on KLH-specific immunity (Sandmaier 1999). In this study, proliferative responses to stimulation with KLH and the KLH-antigen complex were substantially stronger than responses to the synthetic carbohydrate itself in all ovarian cancer patients tested, similar to what has previously been reported for viral vectors.

The use of vaccinia and folwpox viruses as viral vectors was reported by three studies (Gulley 2008; Mohebtash 2009; Odunsi 2007a). Anti-vector immune responses were reported to be investigated by only one of these and occurred in all ovarian cancer patients treated (Gulley 2008).

**Table 7** Definitions and results of anti-idiotypic (Ab2) humoral responses in antigen-specific monoclonal antibody studies

| Study | | N | Dose | target antigen | analysed | positive if: | % positive* | robust if: | % robust* |
|---|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | 2mg | CA-125 | yes | >50ng/ml | 63% | >100ng/ml | |
| Berek | 2004 | 145 | 2mg | CA-125 | yes | | | >100ng/ml | 67% |
| Berek | 2009 | 371 | 2mg | CA-125 | yes | unk | n.r. | unk | n.r. |
| Braly | 2009 | 40 | unk | CA-125 | yes | | | >100ng/ml | 94% vs. 74% |
| Ehlen | 2005 | 13 | 2mg | CA-125 | yes | >50ng/ml | 45% | | |
| Gordon | 2004 | 20 | 2mg | CA-125 | yes | >50ng/ml | | >100ng/ml | 79% |
| Ma | 2002 | 4 | unk | CA-125 | no | | | | |
| Method | 2002 | 102 | 2mg | CA-125 | yes | | | >100ng/ml | 13% vs. 31% vs. 67% |
| Möbus | 2003 | 44 | 2mg | CA-125 | yes | | | >50ng/ml | 77% |
| Nicholson | 2004 | 26 | 25mg | MUC1 | yes | unk | 100% | | |
| Noujaim | 2001 | 184 | 2mg | CA-125 | no | | | | |
| Pfisterer | 2006 | 36 | 2mg | CA-125 | no | | | | |
| Reinartz | 2004 | 119 | 2mg | CA-125 | no | | | | |
| Sabbatini | 2006 | 42 | 2mg/0.2mg | CA-125 | no | | | | |
| Schultes | 1998 | 75 | 2mg | CA-125 | yes | >50ng/ml | 64% | >250ng/ml | |
| Ströhlein | 2009 | 2 | 10/20/40µg<br>10/40/80µg | EpCAM<br>Her2/Neu | no | | | | |
| van Zanten-Przybysz | 2002 | 5 | 50mg | membrane folate receptor | no | | | | |
| Wagner | 1993 | 58 | 1mg | CA-125 | yes | >0u/l | 64% | >10u/l | 32% |

Unk – unknown; n.r. – not reported

**Table 8** Definitions and results of anti-anti-idiotypic (Ab3) humoral responses in antigen-specific monoclonal antibody studies

| Study | | N | Dose | target antigen | analyzed | positive if: | % positive* | robust if: | % robust* |
|---|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | 2mg | CA-125 | no | | | | |
| Berek | 2004 | 145 | 2mg | CA-125 | no | | | | |
| Berek | 2009 | 371 | 2mg | CA-125 | no | | | | |
| Braly | 2009 | 40 | unk | CA-125 | no | | | | |
| Ehlen | 2005 | 13 | 2mg | CA-125 | yes | >100ng/ml | | ≥3x baseline | 0% |
| Gordon | 2004 | 20 | 2mg | CA-125 | yes | >100ng/ml | | ≥3x baseline | 11% |
| Ma | 2002 | 4 | unk | CA-125 | no | | | | |
| Method | 2002 | 102 | 2mg | CA-125 | no | | | | |
| Möbus | 2003 | 44 | 2mg | CA-125 | yes | | | ≥3x baseline | 28% |
| Nicholson | 2004 | 26 | 25mg | MUC1 | yes | >0.015µg/ml | 38% | | |
| Noujaim | 2001 | 184 | 2mg | CA-125 | yes | | | ≥3x baseline | 43% |
| Pfisterer | 2006 | 36 | 2mg | CA-125 | yes | >1000ng/ml | L vs. S: 100% vs. 100% | | |
| Reinartz | 2004 | 119 | 2mg | CA-125 | yes | >1000u/ml | 68% | | |
| Sabbatini | 2006 | 42 | 2mg/0.2mg | CA-125 | yes | >1000u/ml | 100% | | |
| Schultes | 1998 | 75 | 2mg | CA-125 | yes | >200ng/ml | 24% | ≥3x baseline | |
| Ströhlein | 2009 | 2 | 10/20/40µg | EpCAM | no | | | | |
| | | | 10/40/80µg | Her2/Neu | | | | | |
| van Zanten-Przybysz | 2002 | 5 | 50mg | membrane folate receptor | no | | | | |
| Wagner | 1993 | 58 | 1mg | CA-125 | no | | | | |

**Table 9** Definitions and results of humoral response evaluation in other antigen-specific immunotherapy studies

| Study | | N | target antigen(s) | analyzed | assay | positive if: | % positive |
|---|---|---|---|---|---|---|---|
| Brossart | 2008 | 3 | Her-2/Neu, MUC1 | no | | | |
| Chianese-Bullock | 2008 | 9 | FBP, Her-2/Neu, MAGE-A1 | no | | | |
| Chu | 2008 | 14 | Her-2/Neu, hTERT, PADRE | no | | | |
| Diefenbach | 2008 | 9 | NY-ESO-1 | yes | ELISA | >100 | 0% |
| Freedman | 1998 | 30 | Sialyl Tn | no | | | |
| Gulley | 2008 | 3 | CEA, MUC1 | no | | | |
| Herrin | 2007 | 21 | p53 | no | | | |
| Leffers | 2009a | 20 | p53 | yes | unk | unk | Pre-imm.: 40%, post-imm.:45% |
| MacLean | 1992 | 10 | Thomson Friedenreich | yes | ELISA | unknown | 80% IgA, 90% IgM, 90% IgG, 0% IgE |
| MacLean | 1996 | 34 | Sialyl Tn | yes | ELISA | unknown | 96% |
| Mohebtash | 2009 | 17 | MUC1, CEA | no | | | |
| Nishikawa | 2006 | 4 | NY-ESO-1 | no | | | |
| Odunsi | 2007 | 18 | NY-ESO-1 | yes | ELISA | unknown | 22% |
| Odunsi | 2007a | 19 | NY-ESO-1 | yes | ELISA | unknown | 38% |
| Sabbatini | 2000 | 25 | Lewis Y | yes | ELISA | unknown | 67% |
| Sabbatini | 2007 | 11 | GM2, Globo-H, Lewis Y, Tn-MUC1, Tn(c), sTN(c), TF(c) | yes | ELISA | Negative to ≥ 1:40 or 8-fold increase | 89% ≥3 antigens; 22% GM2, 33% Globo-H, 11% Lewis Y, 100% Tn-MUC1, 44% Tn(c), 44% sTN(c), 78% TF(c) |
| Sandmaier | 1999 | 7 | Sialyl Tn | yes | ELISA | ≥1:20 | 100% IgM, 80% IgG |
| Tsuda | 2004 | 5 | patient-tailored cocktail | yes | ELISA | unknown | 67% |

**Table 10** Definitions and results of cellular responses in antigen-specific monoclonal antibody studies

| Study | | N | Dose | target antigen | analyzed | assay | positive if: | % positive* |
|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | 2mg | CA-125 | no | | | |
| Berek | 2004 | 145 | 2mg | CA-125 | no | | | |
| Berek | 2009 | 371 | 2mg | CA-125 | no | | | |
| Braly | 2009 | 40 | unk | CA-125 | yes | ELISPOT | permutation test | simultaneous 44% vs. delayed 21% |
| Ehlen | 2005 | 13 | 2mg | CA-125 | yes | ELISPOT | permutation test | n=4 CA-125: 75%; n=3 oregovomab 67% |
| Gordon | 2004 | 20 | 2mg | CA-125 | yes | ELISPOT | permutation test | n=18 CA-125: 39%; n=18 oregovomab 50%; n=8 autologous tumor cells 63% |
| Ma | 2002 | 4 | unk | CA-125 | yes | proliferation assay | unk | n=4: 50% |
| Method | 2002 | 102 | 2mg | CA-125 | yes | ELISPOT | NR | NR |
| Möbus | 2003 | 44 | 2mg | CA-125 | no | | | |
| Nicholson | 2004 | 26 | 25mg | MUC1 | no | | | |
| Noujaim | 2001 | 184 | 2mg | CA-125 | yes | proliferation assay / cytokine ELISA | proliferation assay: wilcoxon signed rank test; cytokine ELISA: unknown | n=17 CA-125 53%; Th1 cytokines 41%, Th2 cytokines 94% |
| Pfisterer | 2006 | 36 | 2mg | CA-125 | yes | cytokine flow cytometry | >2-fold increase in IFN-γ expressing T-cells | L vs S: n=12 vs 17, CD4: 58% vs 29%; CD8 75% vs 18% |
| Reinartz | 2004 | 119 | 2mg | CA-125 | no | | | |
| Sabbatini | 2006 | 42 | 2mg/0.2mg | CA-125 | yes | ELISPOT | spots experimental wells - control wells >20 & experimental wells/control wells >1.5x | n=5: 80% |
| Schultes | 1998 | 75 | 2mg | CA-125 | no | | | |
| Ströhlein | 2009 | 2 | 10/20/40µg 10/40/80µg | EpCAM Her2/Neu | yes | IFN-γ secretion assay | unk | EpCAM 100% (n=1) Her2/Neu 0% (n=1) |
| van Zanten-Przybysz | 2002 | 5 | 50mg | membrane folate receptor | yes | proliferation assay | unk | 0% |
| Wagner | 1993 | 58 | 1mg | CA-125 | yes | leukocyte migration inhibition assay | unk | 21% |

Unk – unknown; NR – not reported

**Table 11** Definitions and results of cellular responses in other antigen-specific immunotherapy studies

| Study | | N | target antigen(s) | analyzed | assay | positive if: | % positive |
|---|---|---|---|---|---|---|---|
| Brossart | 2008 | 3 | Her-2/Neu, MUC1 | yes | intracellular INF-γ staining (CD8) | unk | n=1: Her-2/Neu 100%; n=2 MUC1 50% |
| Chianese-Bullock | 2008 | 9 | FBP, Her-2/Neu, MAGE-A1 | yes | ELISPOT (CD8) | unk | n=9: FBP 40%, Her-2/neu 83%, MAGE-A1 83% |
| Chu | 2008 | 14 | Her-2/Neu, hTERT, PADRE | yes | ELISPOT | unk | n= 5: 100% hTERT, 60% Her-2/Neu |
| Diefenbach | 2008 | 9 | NY-ESO-1 | yes | ELISPOT / Tetramer staining (CD8) | specific spots > 30 and >3x spots irrelevant control<br>>0.1% tetramer positive CD8-cells | both assays n=9: 67% |
| Freedman | 1998 | 30 | Sialyl Tn | no | | | |
| Gulley | 2008 | 3 | CEA, MUC1 | yes | ELISPOT (CD8) / IFN-γ ELISA (CD4) | ELISPOT: ≥2-fold increase in IFN-γ secreting cells | N=3: 100% CEA |
| Herrin | 2007 | 21 | p53 | yes | ELISPOT | unk | n=13 vs. 7: 69% vs. 71% |
| Leffers | 2009a | 20 | p53 | yes | ELISPOT<br>Proliferation assay<br>Intracellular IFN-γ staining (CD4/CD8) | ELISPOT: specific spots≥10/$10^5$ PBMC and ≥3x than before imm.<br>Proliferation: cpm > 1000/minute, SI≥3 and ≥2x than before imm.<br>Intracellular staining: ≥3x pre-imm. | N=18: 100%<br>N=17: 82%<br>N=5: CD8 0%, CD4 100% |
| MacLean | 1996 | 10 | Sialyl Tn Thomson | no | | | |
| MacLean | 1992 | 34 | Friedenreich | no | | | |
| Mohebtash | 2009 | 17 | MUC1, CEA | no | | | |
| Nishikawa | 2006 | 4 | NY-ESO-1 | yes | ELISPOT (CD4) | unk | n=4: 75% |
| Odunsi | 2007 | 18 | NY-ESO-1 | yes | ELISPOT (CD4 / CD8) | ELISPOT: mean ± 3 SD | n=18: CD4 – 83%; n=9: CD8 – 33% |

*Table 11 - continued*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Odunsi | 2007a | 19 | NY-ESO-1 | yes | ELISPOT (CD4 / CD8) | unk | n=9: CD8 – 55%, CD4 ? |
| Sabbatini | 2000 | 25 | Lewis Y | no | | | |
| Sabbatini | 2007 | 11 | GM2, Globo-H, Lewis Y, Tn-MUC1, Tn©, sTN©, TF© | no | | | |
| Sandmaier | 1999 | 7 | Sialyl Tn | yes | proliferation assay | >upper limit of normals (SI 2.35) | n=4 50%* |
| Tsuda | 2004 | 5 | patient-tailored cocktail | yes | IFN-γ ELISA | unclear | n=2 after 6 vacc. 100%; n=1 after 12 vacc. 100% |

* as measured after at least three immunizations; Unk – unknown; SI – stimulation index; SD – standard deviation

**Table 12** Definitions and results of human-anti-mouse antibody (HAMA) evaluation in antigen-specific monoclonal antibody studies

| Study | | N | Dose | target antigen | analyzed | positive if: | % positive* | robust if: | % robust* |
|---|---|---|---|---|---|---|---|---|---|
| Berek | 2001 | 252 | 2mg | CA-125 | yes | | | >5000ng/ml | 51% |
| Berek | 2004 | 145 | 2mg | CA-125 | yes | >200ng/ml | unk | >5000ng/ml | 59% |
| Berek | 2009 | 371 | 2mg | CA-125 | yes | unk | n.r. | | |
| Braly | 2009 | 40 | unk | CA-125 | yes | unk | simultaneous 100% vs. delayed 80% | >3000ng/ml | simultaneous 88% vs. delayed 74% |
| Ehlen | 2005 | 13 | 2mg | CA-125 | yes | >200ng/ml | 100% | >5000ng/ml | 58% |
| Gordon | 2004 | 20 | 2mg | CA-125 | yes | >200ng/ml | unk | >5000ng/ml | 79% |
| Ma | 2002 | 4 | unk | CA-125 | no | | | | |
| Method | 2002 | 102 | 2mg | CA-125 | yes | >200ng/ml | unk | unk | 4% - 36% - 39% |
| Möbus | 2003 | 44 | 2mg | CA-125 | yes | | | >5000ng/ml | 68% |
| Nicholson | 2004 | 26 | 25mg | MUC1 | no | | | | |
| Noujaim | 2001 | 184 | 2mg | CA-125 | no | | | | |
| Pfisterer | 2006 | 36 | 2mg | CA-125 | yes | >15ng/ml | L vs. S: 94% vs. 100% | | |
| Reinartz | 2004 | 119 | 2mg | CA-125 | yes | >100ng/ml | 78% | | |
| Sabbatini | 2006 | 42 | 2mg/0.2mg | CA-125 | yes | >100ng/ml | 90% | | |
| Schultes | 1998 | 75 | 2mg | CA-125 | yes | >200ng/ml | 90% | | |
| Ströhlein | 2009 | 2 | 10/20/40µg | EpCAM | yes | unk | 100% (n=1) | | |
| van Zanten-Przybysz | 2002 | 5 | 10/40/80µg<br>50mg | Her2/Neu<br>membrane folate receptor | n.a. | | | | |
| Wagner | 1993 | 58 | 1mg | CA-125 | no | | | | |

Unk – unknown; n.a. – not applicable; n.r. – not reported

***Adverse events***

For this review, adverse events were defined as any adverse change in health or side-effect that occurred in a person who participated in the clinical trial while the patient was receiving the treatment, irrespective of whether the event could be attributed to the treatment received.

Although 28 studies mentioned adverse events, sufficiently detailed information on adverse events occurring during the trial was available for only 21/36 studies. Local adverse events were explicitly mentioned for 16 studies all of which used local administration of the vaccine (i.e. intradermal, intramuscular or subcutaneous injection). Although these trials report the presence of injection site reactions, 50% did not further specify the type of local adverse events witnessed. When local adverse events were further specified, these were best summarized as pain at the injection site and local inflammatory responses (erythema, induration, pruritis). In one study 3 out of 30 patients developed a small abscesses and ulceration upon intradermal injection, after which the adjuvant was omitted from the vaccine for these patients (Freedman 1998).

Systemic adverse events occurred in 23 studies and were not observed in 2 studies. For the remaining 11 studies no information on systemic adverse events could be deduced from the manuscript. Autoimmunity was reported by two studies. In one study a patient with strong immunological responses to the vaccine developed a symptomatic hypothyroidism necessitating replacement therapy (Diefenbach 2008). A minor induction of anti-nuclear antibodies (grade I according to Common Terminology Criteria for Adverse Events v3.0 (Trotti 2003)) was described for two patients receiving a multi-peptide vaccine (Chianese-Bullock 2008). Allergic reactions were described for a total of 14 patients (Berek 2009; Braly 2009; Ehlen 2005; MacLean 1992; Möbus 2003; Pfisterer 2006; Ströhlein 2009). Allergic reactions were mild and easily managed, e.g. hypersensitivity, allergic exanthema, and urticaria. When study treatment was continued, this did not result in renewed allergic reactions (Braly 2009; Ehlen 2005; Möbus 2003; Pfisterer 2006).

Other systemic adverse events reported, irrespective of whether attributable to the investigated drug, included hematologic changes (e.g. anaemia, leucopenia), flu-like symptoms (including fatigue, myalgia, arthralgia, headache, fever and chills) and gastrointestinal events (e.g. nausea, vomiting, diarrhoea, and abdominal pain), most of which were classified as grade I or II events. Grade III or IV adverse events were reported by eleven studies. For two studies it was however unclear whether the participating ovarian cancer patients experienced these events (Gulley 2008; Tsuda 2004) and in one study serious adverse events undoubtedly reflected progressive disease (Leffers 2009a). In the one study, which investigated p53-based immunization strategies combined with IL-2, grade III/IV adverse events were observed in 42% of patients in each arm of the study and ascribed to the IL-2

adjuvant, which was thereafter omitted from the regimen for these patients (Herrin 2007). Severe or life-threatening flu-like symptoms and gastro-intestinal events were observed in 7 to 30% of patients in 7 studies investigating monoclonal antibodies targeting CA-125 (Berek 2004; Berek 2009; Braly 2009; Ehlen 2005; Gordon 2004; Pfisterer 2006; Sabbatini 2006). However as no differences in serious adverse events were observed between patients treated with the monoclonal antibody and placebo controls, it is unlikely that these can be attributed to the monoclonal antibody treatment.

## DISCUSSION

The aim of this review was to evaluate clinical and immunological efficacy of antigen-specific active immunotherapy in ovarian cancer, whilst also obtaining an impression of safety and tolerability of this treatment modality. The antigen-specific active immunotherapy described in this review can largely be divided into two strategies (1) the administration of antibodies targeting a specific tumour antigen and (2) the administration of (or parts of) a specific tumour antigen itself. As expected, most studies were non-RCTs.

Antigen-specific humoral and/or cellular immunogenicity of the different interventions showed great variation for both monoclonal antibody studies and trials using other strategies. This variation may at least be partially attributed to the variation in immunological response definitions used by the different studies. It is therefore not possible to reliably compare studies and infer which intervention and/or immunization strategy is most promising for the induction of strong anti-tumour immunity. Furthermore, only two studies evaluated recognition of autologous tumour cells *in vitro* and none evaluated immune responses at the tumour site. Although obtaining autologous tumour material may be burdensome, such assays would be extremely valuable as they comprise true interactions between induced immunity and tumour cells and could as such provide important information on how to continue improvement of immunotherapeutic strategies to reach clinical effectiveness.



Clinical responses to immunotherapy (i.e. tumour responses, responses to secondary treatment and survival benefits) were observed only incidentally and when described reliability of results was questionable due to the absence of clear response definitions. An important comment regarding the likelihood of clinical responses to immunotherapy especially in uncontrolled trials which frequently include patients with recurrent disease, is the fact that this likelihood may be affected by the disease status at start of treatment (Leffers 2009). The indication for immunotherapeutic treatment in the adjuvant setting is supported by the observation of enhanced antigen-specific responses to immunotherapy when combined with chemotherapeutic agents currently or previously used in the primary treatment of ovarian cancer i.e. docetaxel or cyclophosphamide (Garnett 2008; Laheru 2008). Three large randomised controlled studies using a monoclonal CA-125 antibody in the adjuvant setting after successful primary therapy however did not demonstrate any differences in time to relapse and/or OS between the treatment and placebo arm (Berek 2001; Berek 2004;Berek 2009), which indicates that despite immunogenicity, CA-125 targeted monoclonal antibody therapy is (thus far) clinically ineffective. For the studies of other vaccine types, no such conclusions can be made at this time as large controlled studies and more studies in the adjuvant, rather than recurrent setting have yet to be performed for the different strategies.

Adverse events, reported in sufficient detail for interpretation, were reported in almost 60% of studies. A distinction was made between local and systemic events. The latter were further subdivided in autoimmunity, allergy and other adverse events. We did not evaluate whether adverse events could be or were considered attributable to the treatment studied, although for local adverse events this is indisputably the case. Inflammatory reactions and pain at the injection site were frequently reported for studies using intradermal, subcutaneous or intramuscular application. Severe or life-threatening systemic adverse events were reported by eleven studies, seven of which investigated monoclonal antibodies targeting CA-125. For these monoclonal antibody studies, no pattern suggestive of an underlying treatment-associated process could be identified and events were often considered to be associated with ovarian cancer progression. Serious adverse events in another study were considered to be related to IL-2 given as an adjuvant to the antigen-specific active immunization.

A disturbing observation regarding adverse events is the lack of uniformity in adverse event reporting. Reporting of safety and tolerability of new treatment strategies should have high priority in all studies of investigational drugs, especially in uncontrolled phase I and II trials. To promote uniformity in adverse event evaluation and reporting as well as the comparability of adverse events between studies, in addition to the NCI Common Terminology Criteria for Adverse Events (Trotti 2003) the Brighton Collaboration (Brighton Collaboration 2009) has committed itself to develop standardized, widely disseminated and globally accepted case definitions for an exhaustive number of adverse events following immunisation as well as guidelines for data collection, analysis, and presentation. These case-definitions and guidelines are freely available and we strongly recommend that, where applicable, these are used for all immunotherapeutic trials.



Interestingly, for 6 studies described in this review, information from the study was collected from a meeting abstract only and often this meeting abstract was several years old. The lack of full text manuscripts, even after contacting abstract authors, strongly suggests the existence of a publication bias. To avoid the disappearance of negative trials, registration of trials in a prospective trial register is widely recommended and supported by the International Committee of Medical Journal Editors (ICMJE). However, initially in 2005 registration was only requested for controlled trials. Since July 1, 2008 all trials prospectively assigning human participants to one or more health-related interventions to evaluate the effects on health outcomes are required to register in a clinical trial register approved by the WHO. From the ongoing studies section it is however apparent that despite registration in a prospective trial register, studies may suffer from publication bias as several relatively small studies started more than five years ago have not yet been published to date or closed according to the trial register. In addition to registration in trial registers, the uniform requirements for manuscripts submitted to biomedical

journals drafted by the ICMJE encourage uniformity in reporting of clinical trials by stating ethical principles in conduct and reporting of research as well as providing recommendations relating to specific elements of editing and writing. As is obvious from this review, the scientific community might benefit substantially if also early phase uncontrolled clinically trials would strive for uniformity in trial conduct and reporting.

This review also emphasizes another aspect of immunotherapeutic trials that warrants serious attention in the immunotherapeutic scientific community, i.e. the lack of consensus on 1) what assays to use to establish immunogenicity of an intervention (Britten 2008), 2) what cut-offs to use to define true immunological responses, and 3) response definitions for clinical efficacy. Given these large inconsistencies it is evident that the elucidation of what type of immunological response is necessary for and/or a surrogate marker of clinical activity of an immunotherapeutic intervention is burdensome.

In summary, this review describes 36 immunotherapy trials in ovarian cancer patients. The most striking observations of this review unfortunately do not concern the aim of the review, but address the lack of uniformity in conduct and reporting of early phase immunotherapy trials. When temporarily discarding this methodological heterogeneity, it seems that although all strategies described are capable of inducing immunological responses, be it humoral or cellular, clinically effectiveness thus far has not been convincingly demonstrated. The largest body of evidence is available for CA-125 directed antibody therapy, which has been studied in 1505 patients participating in 15 studies. As complete or partial clinical responses were reported in only one study and three large RCTs did not demonstrate any clinical benefit of antibody treatment, we feel that it is unlikely that clinical effectiveness of CA-125 directed antibody therapy for ovarian cancer will ever be obtained. However, in view of the immunological responses to and the usually mild side-effects, we feel that further investigation of other antigen-specific active immunotherapy strategies in ovarian cancer is worthwhile.

## AUTHORS' CONCLUSIONS

### Implications for practice

At this point in time, there is no effective immunotherapy for ovarian cancer. Although promising immunological responses have been observed for most strategies evaluated, these do not coincide with clinical benefits for ovarian cancer patients. Until evidence of true clinical effectiveness is available, immunotherapy should therefore not be offered as an alternative to standard therapy for primary or recurrent ovarian cancer.

### Implications for research

Our primary recommendation relates to the necessity of uniformity in trial conduct and reporting. Not until universally accepted immunological and clinical response definitions and guidelines for adverse events reporting are adopted in immunotherapeutic trials, will it be possible to make any inferences about the achievability of immunotherapy as a treatment for ovarian cancer. Furthermore, expanding evaluation of immunogenicity to include recognition of autologous tumour is advisable. Given the usually mild side-effects and the immunological responses witnessed in most trials, we feel that further investigation of antigen-specific active immunotherapy in ovarian cancer is worth wile.

**Appendex 1** PubMed search strategy

PubMed RCT search filter:

1. randomised controlled trial [pt]
2. controlled clinical trial [pt]
3. randomised [tiab]
4. placebo [tiab]
5. drug therapy [sh]
6. randomly [tiab]
7. trial [tiab]
8. groups [tiab]
9. #1 or #2 or #3 or #4 or #5 or #6 or #7 or #8
10. humans [mh]
11. #9 and #10

PubMed search for patient population:
12. ovary
13. ovarian
14. # 12 OR # 13
15. cancer OR carcinoma OR neoplasm OR tumor OR tumors OR tumour OR tumours OR malignan*
16. # 14 AND # 15
17. ovarian neoplasms [mh]
18. # 16 OR # 17

PubMed search for Intervention:
19. immunotherapy[tiab]
20. vaccination[tiab]
21. vaccine[tiab]
22. immunization[tiab]
23. active immunotherapy[mh]
24. cancer vaccines[mh]
25. #19 OR #20 OR #21 OR #22 OR #23 OR #24
26. antigen* OR tumor antigen OR tumour antigen
27. Antigens, Neoplasm [mh]
28. # 26 OR # 27
29. T cell OR T-cell OR T lymphocyte OR T-lymphocyte OR CD4-positive T-lymphocyte OR CD8-positive lymphocyte
30. T-lymphocytes [mh]
31. # 29 OR # 30
32. # 25 OR # 28 OR # 31

Search for all types of different trials:
33. # 18 AND # 32 (all trials)
34. #33 AND #10 (all human trials)
35. # 11 AND # 18 AND # 32 (RCT's only) *

**Appendix 2** EMBASE search strategy

1. 'crossover procedure'/exp
2. 'double-blind procedure'/exp
3. 'randomized controlled trial'/exp
4. 'single-blind procedure'/exp
5. random$ OR crossover$ OR 'cross over'$ OR cross AND over$ OR factorial$ OR placebo$ OR doubl$ adj blind$ OR singl$ adj blind$ OR allocat$ OR assign$ OR volunteer$
6. #1 OR #2 OR #3 OR #4 OR #5
7. 'ovary' OR 'ovarian' OR 'ovarium'
8. 'cancer' OR 'carcinoma' OR 'neoplasm' OR 'tumor' OR 'tumour' OR 'tumors' OR 'tumours' OR 'malignancy'
9. #7 AND #8
10. 'ovary tumor'/exp
11. #9 OR #10
12. 'active immunization'/exp
13. 'cancer immunization'/exp
14. 'cancer vaccine'/exp
15. 'vaccination' OR 'vaccine' OR 'immunization' OR 'immunisation' OR 'immunotherapy'
16. #12 OR #13 OR #14 OR #15
17. 'tumor antigen'/exp
18. 't cell' OR 't-cell' OR 't lymphocyte' OR 't-lymphocyte' OR 'cd4-positive t-lymphocyte' OR 'cd8-positive t-lymphocyte'
19. 't lymphocyte'/exp
20. #18 OR #19
21. #16 AND #17 AND #20
22. #11 AND #21 AND [humans]/lim

**Appendix 3** CENTRAL search strategy

(ovary OR ovarian) AND ((cancer OR carcinoma OR neoplasm OR tumor OR tumors OR tumour OR tumours OR malignan*) OR (ovarian neoplasms))
and
(immunotherapy OR vaccination OR vaccine OR immunization OR active immunotherapy OR cancer vaccines)
and
(antigen* OR tumor antigen OR tumour antigen) OR (Antigens Neoplasm)
and
(T cell OR T-cell OR T lymphocyte OR T-lymphocyte OR CD4-positive T-lymphocyte OR CD8-positive lymphocyte)

## REFERENCES TO STUDIES

### Included studies

***Berek 2001***
Berek JS, Ehlen TG, Gordon A, Nicodemus CF, Schultes B, Whiteside TL et al. Interim analysis of a double blind study of Ovared mAb B43.13 (OV) versus placebo (PBO) in patients with ovarian cancer. In: American Society of Clinical Oncology Annual Meeting. 2001.

***Berek 2004***
Berek JS, Taylor PT, Gordon A, Cunningham MJ, Finkler N, Orr J et al. Randomized, placebo-controlled study of oregovomab for consolidation of clinical remission in patients with advanced ovarian cancer. Journal of Clinical Oncology 2004;22(17):3507-16.

Berek JS, Taylor PT, Nicodemus CF. CA125 velocity at relapse is a highly significant predictor of survival post relapse: results of a 5-year follow-up survey to a randomized placebo-controlled study of maintenance oregovomab immunotherapy in advanced ovarian cancer. Journal of Immunotherapy 2008;31(2):207-14.

***Berek 2009***
Berek J, Taylor P, McGuire W, Smith LM, Schultes B, Nicodemus CF. Oregovomab maintenance monoimmunotherapy does not improve outcome in advanced ovarian cancer. Journal of Clinical Oncology 2009;27:418-25.

Berek J, Taylor PT, McGuire WP, Smith LM, Schultes B, Nicodemus CF. Evaluation of maintenance mono-immunotherapy to improve outcomes in advanced ovarian cancer (OV CA). In: 2008 ASCO Annual Meeting Proceedings. 2008.

***Braly 2009***
Braly P, Chu C, Collins Y, Edwards R, Gordon A, McGuire W, et al. Prospective evaluation of front-line chemo-immunotherapy (C-IT) with oregovomab (2 alternative dosing schedules) carboplatin-paclitaxel (C-P) in advanced ovarian cancer (OC). In: American Society of Clinical Oncology Annual Meeting. 2007.

Braly P, Nicodemus CF, Chu C, Collins Y, Edwards R, Gordon A, McGuire W, Schoonmaker C, Whiteside T, Smith LM, Method M. The immune adjuvant properties of front-line carboplatin-paclitaxel: a randomised phase 2 study of alternative schedules of intravenous oregovomab chemoimmunotherapy in advanced ovarian cancer. Journal of Immunotherapy 2009;32:54-65.

Method M, Gordon A, Smith LM, Nicodemus CF. Oregovomab immune-modulating antibody therapy concurrent tot standard chemotherapy of epithelial ovarian cancer (EOC): feasibility and initial clinical experience. In: Society of Gynecologic Oncologists Annual Meeting on Women's Cancer. 2006.

***Brossart 2000***
Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 2000;96(9):3102-8.

***Chianese-Bullock 2008***
Chianese-Bullock KA, Irvin WP, Jr., Petroni GR, Murphy C, Smolkin M et al. A multipeptide vaccine is safe and elicits T-cell responses in participants with advanced stage ovarian cancer. Journal of Immunotherapy 2008;31(4):420-30.

***Chu 2008***
Chu CS, Boyer J, Coukos G, Rubin SC, Morgan MA, Bendig DL et al. Autologous dendritic cell (IDD-6) vaccination as consolidation for advanced ovarian cancer. In: Society of Gynecologic Oncologists: Annual Meeting on Women's Cancer. 2008.

***Diefenbach 2008***
Diefenbach CSM, Gnjatic S, Sabbatini P, Aghajanian C, Hensley ML, Spriggs DR et al. Safety and Immunogenicity Study of NY-ESO-1b Peptide and Montanide ISA-51 Vaccination of Patients with Epithelial Ovarian Cancer in High-Risk First Remission. Clinical Cancer Research 2008;14(9):2740-8.

***Ehlen 2005***
Ehlen TG, Hoskins PJ, Miller D, Whiteside TL, Nicodemus CF, Schultes BC et al. A pilot phase 2 study of oregovomab murine monoclonal antibody to CA125 as an immunotherapeutic agent for recurrent ovarian cancer. International Journal of Gynecological Cancer 2005;15(6):1023-34.

***Freedman 1998***
Freedman RS, Kudelka AP, Verschraegen CF, Edwards CL, Tomasovic B, Kaplan A et al. Therapeutic anti-cancer vaccine: a randomized double blind dose comparison study of sialyl Tn-KLH with Detox-B SE adjuvant for active specific immunotherapy of ovarian cancer (OC). In: American Society of Clinical Oncology Annual Meeting. 1998.

Termrungruanglert W, Kudelka AP, Verschraegen CF, Freedman RS, Edwards CL, Tomasovic B et al. Therapeutic anticancer vaccine: a randomized double blind dose comparison study of sialyl TN-K with detox-B SE adjuvant for active specific immunotherapy of ovarian cancer (OC). In: American Society of Clinical Oncology Annual Meeting. 1996.

***Gordon 2004***
Gordon AN, Schultes BC, Gallion H, Edwards R, Whiteside TL, Cermak JM et al. CA125- and tumor-specific T-cell responses correlate with prolonged survival in oregovomab-treated recurrent ovarian cancer patients. Gynecologic Oncology 2004;94(2):340-51.

***Gulley 2008***
Gulley JL, Arlen PM, Tsang KY, Yokokawa J, Palena C, Poole DJ, Remondo C, Cereda V, Jones JL, Pazdur MP, Higgins JP, Hodge JW, Steinberg SM, Kotz H, Dahut WL, Schlom J. Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with metastatic carcinoma. Clinical Cancer Research 2008;14:3060-9.

***Herrin 2007***
Herrin V, Achtar M, Steinberg S, Whiteside TL, Wieckowsk E, Czystowska M et al. A randomized phase II p53 vaccine trial comparing subcutaneous direct administration with intravenous peptide-pulsed dendritic cells in high risk ovarian cancer patients. In: American Society of Clinical Oncology Annual Meeting. 2007.

Herrin V, Behrens RJ, Achtar M, Monahan B, Bernstein S, Brent-Steele T et al. Wild-type p53 peptide vaccine can generate a specific immune response in low burden ovarian adenocarcinom. In: American Society of Clinical Oncology Annual Meeting. 2003.

***Leffers 2009a***
Leffers N, Lambeck AJ, Gooden MJ, Hoogeboom BN, Wolf R, Hamming IE, Hepkema BG, Willemse PH, Molmans BH, Hollema H, Drijfhout JW, Sluiter WJ, Valentijn AR, Fathers LM, Oostendorp J, van der Zee AG, Melief CJ, van der Burg SH, Daemen T, Nijman HW. Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial. Int J Cancer. May 28, 2009 (epub ahead of print).

***Ma 2002***
Ma J, Zhou L, Wang D. Functional mimicry of an anti-idiotypic antibody to nominal antigen on cellular response. Japanese Journal of Cancer Research 2002;93(1):78-84.

***MacLean 1992***
MacLean GD, Bowen-Yacyshyn MB, Samuel J, Meikle A, Stuart G, Nation J, et al. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. Journal of Immunotherapy 1992;11(4):292-305.

***MacLean 1996***
MacLean GD, Reddish MA, Koganty RR, Longenecker BM. Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic STn vaccine. Journal of Immunotherapy With Emphasis on Tumor Immunology 1996;19(1):59-68.

***Method 2002***
Method M, Gordon A, Finkler N, Fingert H, Nicodemus CF, Whiteside TL et al. Randomized evaluation of 3 treatment schedules to optimize clinical activity of OvaRex Mab B43.13 (OV) in patients (pts) with epithelial ovarian cancer (EOC). In: American Society of Clinical Oncology Annual Meeting. 2002.

***Mohebtash 2009***
Mohebtash M, Madan R, Tsang K, Arlen Ph, Pazdur M, Jones J, Schlom J, Gulley J. Clinical outcomes following immunotherapy with a MUC1/CEA vaccine in patients with metastatic breast and ovarian cancer. In: American Association of Cancer Research Annual Meeting. 2009.

***Möbus 2003***
Mobus VJ, Baum RP, Bolle M, Kreienberg R, Noujaim AA, Schultes BC et al. Immune responses to murine monoclonal antibody-B43.13 correlate with prolonged survival of women with recurrent ovarian cancer. American Journal of Obstetrics and Gynecology 2003;189(1):28-36.

***Nicholson 2004***
Nicholson S, Bomphray CC, Thomas H, McIndoe A, Barton D, Gore M et al. A phase I trial of idiotypic vaccination with HMFG1 in ovarian cancer. Cancer Immunology, Immunotherapy 2004;53(9):809-16.

***Nishikawa 2006***
Nishikawa H, Qian F, Tsuji T, Ritter G, Old LJ, Gnjatic S et al. Influence of CD4(+)CD25(+) regulatory T cells on low/high-avidity CD4(+) T cells following peptide vaccination. Journal of Immunology 2006;176(10):6340-6.

***Noujaim 2001***
Noujaim AA, Schultes BC, Baum RP, Madiyalakan R. Induction of CA125-specific B and T cell responses in patients injected with MAb-B43.13--evidence for antibody-mediated antigen-processing and presentation of CA125 in vivo. Cancer Biotherapy & Radiopharmaceuticals 2001;16(3):187-203.

***Odunsi 2007***
Odunsi K, Qian F, Matsuzaki J, Mhawech-Fauceglia P, Andrews C, Hoffman EW et al. Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. Proceedings of the National Academy of Sciences of the United States of America 2007;104(31):12837-42.

***Odunsi 2007a***
Odunsi K, Rodabaugh K, Lele S, Old LJ, Matsuzaki J, Qian F et al. Diversified prime and boost vaccination using recombinant vaccinia and fowlpox expressing NY-ESO-1 efficiently induces antibody, CD4+, and CD8+ antitumor immune responses in patients with ovarian cancer. In: Society of Gynecologic Oncologists Annual Meeting on Women's Cancer. 2007.

***Pfisterer 2006***
Pfisterer J, du BA, Sehouli J, Loibl S, Reinartz S, Reuss A et al. The anti-idiotypic antibody abagovomab in patients with recurrent ovarian cancer. A phase I trial of the AGO-OVAR. Annals of Oncology 2006;17(10):1568-77.

***Reinartz 2004***
Reinartz S, Kohler S, Schlebusch H, Krista K, Giffels P, Renke K et al. Vaccination of patients with advanced ovarian carcinoma with the anti-idiotype ACA125: immunological response and survival (phase Ib/II). Clinical Cancer Research 2004;10(5):1580-7.

Wagner U, Kohler S, Reinartz S, Giffels P, Huober J, Renke K et al. Immunological consolidation of ovarian carcinoma recurrences with monoclonal anti-idiotype antibody ACA125: immune responses and survival in palliative treatment.. Clinical Cancer Research 2001;7(5):1154-62.

***Sabbatini 2000***
Sabbatini PJ, Kudryashov V, Ragupathi G, Danishefsky SJ, Livingston PO, Bornmann W et al. Immunization of ovarian cancer patients with a synthetic Lewis(y)-protein conjugate vaccine: a phase 1 trial. Clinical Cancer Research 2000;87(1):79-85.

***Sabbatini 2006***
Sabbatini P, Dupont J, Aghajanian C, Derosa F, Poynor E, Anderson S et al. Phase I study of abagovomab in patients with epithelial ovarian, fallopian tube, or primary peritoneal cancer. International Journal of Cancer 2006;12(18):5503-10.

***Sabbatini 2007***
Sabbatini PJ, Ragupathi G, Hood C, Aghajanian CA, Juretzka M, Iasonos A, Hensley ML, Spassova MK, Ouerfelli O, Spriggs DR, Tew WP, Konner J, Clausen H, Abu Rustum N, Dansihefsky SJ, Livingston PO. Pilot study of a heptavalent vaccine-keyhole limpet hemocyanin

conjugate plus QS21 in patients with epithelial ovarian, fallopian tube, or peritoneal cancer. Clinical Cancer Research 2007;13:4170-7.

***Sandmaier 1999***
Sandmaier BM, Oparin DV, Holmberg LA, Reddish MA, MacLean GD, Longenecker BM. Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cell rescue, and immunization with Theratope STn-KLH cancer vaccine. Journal of Immunotherapy 1999;22(1):54-66.

***Schultes 1998***
Schultes B, Yang R, Agopsowicz K, Kuzma M, Dharampaul S, Baum R et al. Anti-idiotype induction therapy for ovarian cancer: immune responses in patients injected with OvaRex-Mab-B43.13. In: American Society of Clinical Oncology Annual Meeting. 1999.

Schultes BC, Baum RP, Niesen A, Noujaim AA, Madiyalakan R. Anti-idiotype induction therapy: anti-CA125 antibodies (Ab3) mediated tumor killing in patients treated with Ovarex mAb B43.13 (Ab1). Cancer Immunol Immunother 1998;46(4):201-12.

***Ströhlein 2009***
Ströhlein MA, Siegel R, Jäger M, Lindhofer H, Jauch KW, Heiss MM. Induction of anti-tumor immunity by trifunctional antibodies in patients with peritoneal carcinomatosis. Journal of Experimental & Clinical Cancer Research 2009;28:18.

***Tsuda 2004***
Tsuda N, Mochizuki K, Harada M, Sukehiro A, Kawano K, Yamada A et al. Vaccination with predesignated or evidence-based peptides for patients with recurrent gynecologic cancers. Journal of Immunotherapy 2004;27(1):60-72.

***van Zanten-Przybysz 2002***
van Zanten-Przybysz I, Molthoff C, Gebbinck JK, von Mensdorff-Pouilly S, Verstraeten R, Kenemans P et al. Cellular and humoral responses after multiple injections of unconjugated chimeric monoclonal antibody MOv18 in ovarian cancer patients: a pilot study. Journal of Cancer Research and Clinical Oncology 2002;128(9):484-92.

***Wagner 1993***
Wagner U, Reinsberg J, Schmidt S, Mallmann P, Schmolling J, Schultes B et al. Monoclonal antibodies and idiotypic network activation for ovarian carcinoma. Cell Biophysics 1994;24-25:237-42.

Wagner U. Antitumor antibodies for immunotherapy of ovarian carcinomas. Hybridoma 1993;12(5):521-8.

## Excluded studies

***Anderson 2000***
Anderson BW, Kudelka AP, Honda T, Pollack MS, Gershenson DM, Gillogly MA et al. Induction of determinant spreading and of Th1 responses by in vitro stimulation with HER-2 peptides. Cancer Immunology, Immunotherapy 2000;49(9):459-68.

***Bender 2007***
Bender A, Karbach J, Neumann A, Jager D, Al-Batran SE, Atmaca A et al. LUD 00-009: Phase 1 study of intensive course immunization with NY-ESO-1 peptides in HLA-A2 positive patients with NY-ESO-1-expressing cancer. Cancer Immunology, Immunotherapy 2007;7:16.

***Carbone 2005***
Carbone DP, Ciernik IF, Kelley MJ, Smith MC, Nadaf S, Kavanaugh D et al. Immunization with mutant p53- and K-ras-derived peptides in cancer patients: immune response and clinical outcome. Journal of Clinical Oncology 2005;23(22):5099-107.

***Disis 1999***
Disis ML, Grabstein KH, Sleath PR, Cheever MA. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clinical Cancer Research 1999;5(6):1289-97.

***Disis 2000***
Disis ML, Schiffman K, Gooley TA, McNeel DG, Rinn K, Knutson KL. Delayed-type hypersensitivity response is a predictor of peripheral blood T-cell immunity after HER-2/neu peptide immunization. Clinical Cancer Research 2000;6(4):1347-50.

***Disis 2002***
Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA et al. Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. Journal of Clinical Oncology 2002;20(11):2624-32.

***Disis 2002a***
Disis ML, Rinn K, Knutson KL, Davis D, Caron D, dela RC et al. Flt3 ligand as a vaccine adjuvant in association with HER-2/neu peptide-based vaccines in patients with HER-2/neu-overexpressing cancers. Blood 2002;99(8):2845-50.

***Disis 2004***
Disis ML, Goodell V, Schiffman K, Knutson KL. Humoral epitope-spreading following immunization with a HER-2/neu peptide based vaccine in cancer patients. Journal of Clinical Immunology 2004;24(5):571-8.

***Disis 2004a***
Disis ML, Schiffman K, Guthrie K, Salazar LG, Knutson KL, Goodell V et al. Effect of dose on immune response in patients vaccinated with an her-2/neu intracellular domain protein--based vaccine. Journal of Clinical Oncology 2004;22(10):1916-25.

***Hernando 2002***
Hernando JJ, Park TW, Kubler K, Offergeld R, Schlebusch H, Bauknecht T. Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced gynaecological malignancies: clinical and immunological evaluation of a phase I trial. Cancer Immunology, Immunotherapy 2002;51(1):45-52.

***Hernando 2007***
Hernando JJ, Park T-W, Fischer H-P, Zivanovic O, Braun M, Polcher M et al. Vaccination with dendritic cells transfected with mRNA-encoded folate-receptor-(alpha) for relapsed metastatic ovarian cancer. Lancet Oncology 2007;8(5):451-4.

***Holmberg 2000***
Holmberg LA, Oparin DV, Gooley T, Lilleby K, Bensinger W, Reddish MA et al. Clinical outcome of breast and ovarian cancer patients treated with high-dose chemotherapy, autologous stem cell rescue and THERATOPE STn-KLH cancer vaccine. Bone Marrow Transplant 2000;25(12):1233-41.

***Hui 1997***
Hui KM, Ang PT, Huang L, Tay SK. Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA-liposome complexes. Gene Therapy 1997;4(8):783-90.

***Jager 2006***
Jager E, Karbach J, Gnjatic S, Neumann A, Bender A, Valmori D et al. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. Proceedings of the National Academy of Sciences of the United States of America 2006;103(39):14453-8.

***Knutson 2001***
Knutson KL, Schiffman K, Disis ML. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. Journal of Clinical Investigation 2001;107(4):477-84.

***Knutson 2002***
Knutson KL, Schiffman K, Cheever MA, Disis ML. Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369-377, results in short-lived peptide-specific immunity. Clin Cancer Res 2002;8(5):1014-8.

***Letsch 2008***
Letsch A, Asemissen AM, Zimmermann K, Bauer S, Stather D, Völker-Call M, Thiel E, Scheibenbogen C, Keilholz U. Different quality of T cell repsonses to WT1 peptide vaccination in patients with AML/MDS and patients with solid tumors. In: J Immunotherapy. Vol. 31. 2008:943-4.

***Loveland 2006***
Loveland BE, Zhao A, White S, Gan H, Hamilton K, Xing PX et al. Mannan-MUC1-pulsed dendritic cell immunotherapy: a phase I trial in patients with adenocarcinoma. Clinical Cancer Research 2006;12(3 Pt 1):869-77.

***Marshall 2005***
Marshall JL, Gulley JL, Arlen PM, Beetham PK, Tsang KY, Slack R, Hodge JW, Doren S, Grosenbach DW, Hwang J, Fox E, Odogwu L, Park S, Panicali D, Schlom J. Phase I study of sequential vaccinations with fowlpox-CEA(6D)-TRICOM alone and sequentially with vaccinia-CEA(6D)-TRICOM, with and without granulocyte-macrophage colony-stimulating factor, in patients with carcinoembryonic antigen-expressing carcinomas. Journal of Clinical Oncology 2005;23:720-31.

***Miotti 1999***
Miotti S, Negri DR, Valota O, Calabrese M, Bolhuis RL, Gratama JW et al. Level of anti-mouse-antibody response induced by bi-specific monoclonal antibody OC/TR in ovarian-carcinoma patients is associated with longer survival. International Journal of Cancer 1999;84(1):62-8.

***Morse 1999***
Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S et al. A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clinical Cancer Research 1999;5(6):1331-8.

***Morse 2003***
Morse MA, Clay TM, Colling K, Hobeika A, Grabstein K, Cheever MA, Lyerly HK. Her2 dendritic cell vaccines. Clinical Breast Cancer 2003;3, suppl. 4:S164-72.

***Murray 2002***
Murray JL, Gillogly ME, Przepiorka D, Brewer H, Ibrahim NK, Booser DJ et al. Toxicity, immunogenicity, and induction of E75-specific tumor-lytic CTLs by HER-2 peptide E75 (369-377) combined with granulocyte macrophage colony-stimulating factor in HLA-A2+ patients with metastatic breast and ovarian cancer. Clinical Cancer Research 2002;8(11):3407-18.

***Parkhurst 2004***
Parkhurst MR, Riley JP, Igarashi T, Li Y, Robbins PF, Rosenberg SA. Immunization of patients with the hTERT:540-548 peptide induces peptide-reactive T lymphocytes that do not recognize tumors endogenously expressing telomerase. Clinical Cancer Research 2004;10(14):4688-98.

***Reddish 1996***
Reddish MA, MacLean GD, Poppema S, Berg A, Longenecker BM. Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and CD69+ lymphocytes correlate with effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. Cancer Immunology, Immunotherapy 1996;42(5):303-9.

***Salazar 2006***
Salazar LG, Murray JL, Disis ML, Cheever M. A phase I vaccine trial of a HER-2/neu peptide incorporated into PLG microspheres in patients with advanced stage HER2-expressing cancers.. In: ASCO Annual Meeting. 2006.

***Schiffman 2002***
Schiffman K, Rinn K, Disis ML. Delayed type hypersensitivity response to recall antigens does not accurately reflect immune competence in advanced stage breast cancer patients. Breast Cancer Research and Treatment 2002;74(1):17-23.

***Yacyshyn 1995***
Yacyshyn MB, Poppema S, Berg A, MacLean GD, Reddish MA, Meikle A et al. CD69+ and HLA-DR+ activation antigens on peripheral blood lymphocyte populations in metastatic breast and ovarian cancer patients: correlations with survival following active specific immunotherapy. International Journal of Cancer 1995;61(4):470-4.

***Zaks 1998***
Zaks TZ, Rosenberg SA. Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. Cancer Research 1998;58(21):4902-8.

## Ongoing studies

***NCT00017537***
Phase IB Trial of Active Specific Immunotherapy With MVF-HER-2(628-647) and CRL1005 Copolymer Adjuvant in Patients With Metastatic Cancer.

***NCT00019084***
Vaccine therapy with tumor specific mutated p53 or ras peptides alone or in combination with cellular immunotherapy with peptide activated lymphocytes (PAL cells) along with subcutaneous IL-2.

***NCT00019916***
Vaccine Therapy With Tumor Specific p53 Peptides in Adult Patients With Adenocarcinoma of the Breast or Ovary.

***NCT00023634***
An Early Phase Study of an EGFRvIII Peptide Based Vaccine in Patients With EGFRvIII Expressing Cancers.

***NCT00034138***
A Comparative Pharmacokinetics and Safety Study of OvaRex MAb-B43.13 in Patients With Ovarian Epithelial Carcinoma.

***NCT00034372***
Multicenter Clinical Trial of Intravenous OvaRex MAb-B43.13 as Post-Chemotherapy Consolidation for Ovarian Carcinoma.

***NCT00091000***
An Open Label Pilot Study to Evaluate the Safety and Tolerability of PANVAC-V (Vaccinia) and PANVAC-F (Fowlpox) in Combination With Sargramostim in Adults With Metastatic Carcinoma.

***NCT00373217***
Evaluation of the Immunogenicity of Vaccination With Synthetic Peptides in Adjuvant in Patients With Advanced Ovarian, Primary Peritoneal, or Fallopian Tube Cancer.

***NCT00381173***
A Phase 1 Open-Label Study of the Safety and Feasibility of ZYC300 Administration With Cyclophosphamide Pre-Dosing.

***NCT00418574***
Efficacy Multicentre Trial of ImmunoTherapy Vaccination With Abagovomab to Treat Ovarian Cancer Patients (MIMOSA).

***NCT00437502***
A Phase I Study of Ovarian Cancer Peptides Plus GM-CSF and Adjuvant (Montanide ISA-51) as Consolidation Following Optimal Debulking and Systemic Chemotherapy for Women With Advanced Stage Ovarian, Tubal, or Peritoneal Cancer.

***NCT00585845***
Study of Safety and Tolerability of Intravenous CRS-207 in Adults With Selected Advanced Solid Tumors Who Have Failed or Who Are Not Candidates for Standard Treatment.

***NCT00616941***
A Phase I Study of NY-ESO-1 Overlapping Peptides (OLP4) With or Without Immunoadjuvants Montanide and Poly-ICLC Vaccination of Epithelial Ovarian Cancer (EOC), Fallopian Tube, or Primary Peritoneal Cancer Patients in Second or Third Remission.

***NCT00648102***
A Phase I, Open-Label, Dose-Escalation, Multidose Study of CDX-1307, a Mannose Receptor-Targeted hCG-? Vaccine, in Patients With Incurable Locally Advanced or Metastatic Breast, Colorectal, Pancreatic, Bladder and Ovarian Cancer.

***NCT00693342***
A Randomized Phase III Trial in Patients With Epithelial Ovarian, Fallopian Tube or Peritoneal Cancer With a Polyvalent Vaccine-KLH Conjugate + OPT-821 Versus OPT-821.

***NCT00709462***
A Phase I, Open-Label, Dose-Escalation, Multidose Study of CDX-1307, a Mannose Receptor-Targeted hCG-β Vaccine, in Patients With Incurable Breast, Colorectal, Pancreatic, Ovarian or Bladder Cancer (CDX-1307-01).

***NCT00803569***
Phase I Study of ALVAC(2)-NY-ESO-1(M)/TRICOM in Patients With Epithelial Ovarian, Fallopian Tube or Primary Peritoneal Carcinoma Whose Tumors Express NY-ESO-1 or LAGE-1 Antigen.

***NCT00844506***
p53 Synthetic Long Peptides Vaccine With Cyclophosphamide for Ovarian Cancer a Phase II Trial.

***NCT00857545***
A Phase III Randomized, Double-Blind Trial of a Polyvalent Vaccine-KLH Conjugate (NSC 748933) + OPT-821 Versus OPT-821 in Patients With Epithelial Ovarian, Fallopian Tube, or Peritoneal Cancer Who Are in Second or Third Complete Remission.

***NCT00887016***
Open Label Phase I Study to Evaluate the Safety and Tolerability of Vaccine (GI-6207) Consisting of Whole, Heat-Killed Recombinant Saccharomyces Cerevisiae Genetically Modified to Express CEA Protein in Adults With Metastatic CEA-Expressing Carcinoma.

***NCT00887796***
A Phase I Clinical Trial of NY-ESO-1 Protein Immunization in Combination With 5-AZA-2'-Deoxycytidine (Decitabine) in Patients Receiving Liposomal Doxorubicin for Recurrent Epithelial Ovarian or Primary Peritoneal Carcinoma.

## Additional references

***Agarwal 2006***
Agarwal R, Linch M, Kaye SB. Novel therapeutic agents in ovarian cancer. European Journal of Surgical Oncology 2006;32(8):875-86.

***Antonia 2006***
Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clinical Cancer Research 2006;12(3):878-87.

***Benedet 2000***
Benedet JL, Bender H, Jones H 3rd, Ngan HY, Pecorelli S. FIGO staging classifications and clinical practice guidelines in the management of gynecologic cancers. FIGO Committee on Gynecologic Oncology. International Journal of Gynaecology and Obstetrics 2000;70(2):209-62.

***Brighton Collaboration 2009***
Brighton Collaboration. www.brightoncollaboration.org.

***Britten 2008***
Britten CM, Gouttefangeas C, Welters MJ, Pawelec G, Koch S, Ottensmeier C et al. The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. Cancer Immunology, Immunotherapy 2008;57(3):289-302.

***Deeks 2003***
Deeks JJ, Dinnes J, D'Amico R, Sowden AJ, Sakarovitch C, Song F et al. International Stroke Trial Collaborative Group, European Carotid Surgery Trial Collaborative Group. Evaluating non-randomised intervention studies. Health Technology Assessment 2003;7(27):1-173.

***Dong 2006***
Dong HP, Elstrand MB, Holth A, Silins I, Berner A, Trope CG et al. NK- and B-cell infiltration correlates with worse outcome in metastatic ovarian carcinoma. American Journal of Clinical Pathology 2006;125(3):451-8.

***Garnett 2008***
Garnett CT, Schlom J, Hodge JW. Combination of docetaxel and recombinant vaccine enhances T-cell responses and antitumor activity: effects of docetaxel on immune enhancement. Clinical Cancer Research 2008;14(11):3536-44.

***Laheru 2008***
Laheru D, Lutz E, Burke J, Biedrzycki B, Solt S, Onners B. Allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor immunotherapy alone or in sequence with cyclophosphamide for metastatic pancreatic cancer: a pilot study of safety, feasibility, and immune activation. Clinical Cancer Research 2008;14(5):1455–63.

***Leffers 2009***
Leffers N, Daemen CAHH, van der Zee AGJ, Nijman HW. Multimodality treatment warranted for ovarian cancer: immunotherapy, a prerequisite to improve prognosis for this vicious disease. Immunotherapy 2009;1(2):163-5.

***Parkin 2006***
Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA: a Cancer Journal for Clinicians 2005;55(2):74-108.

***Raspollini 2005***
Raspollini MR, Castiglione F, Rossi Degl'innocenti D, Amunni G, Villanucci A et al. Tumour-infiltrating gamma/delta T-lymphocytes are correlated with a brief disease-free interval in advanced ovarian serous carcinoma. Annals of Oncology 2005;16(4):590-6.

***Rustin 2004***
Rustin GJ, Quinn M, Thigpen T; du Bois A, Pujade-Lauraine E, Jakobsen A et al. Re: New guidelines to evaluate the response to treatment in solid tumors (ovarian cancer). Journal of the National Cancer Institute 2004;96(6):487-8.

***Sato 2005***
Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favourable prognosis in ovarian cancer. Proceedings of the National Academy of Sciences of the United States of America 2005;102(51):18538-43.

***Therasse 2000***
Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. Journal of the National Cancer Institute 2000;92(3):205-16.

***Thigpen 2000***
Thigpen JT. Chemotherapy for advanced ovarian cancer: overview of randomized trials. Seminars in Oncology 2000;27(3 Suppl 7):11-6.

***Trotti 2003***
Trotti A, Colevas A, Setser V, Rusch D, Jaques V, Budach C et al. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. Seminars in Radiation Oncology 2003;13(3):176-181A.

***Verhagen 1998***
Verhagen AP, de Vet HC, de Bie RA, Kessels AG, Boers M, Bouter LM et al. The Delphi list: a criteria list for quality assessment of randomized clinical trials for conducting systematic reviews developed by Delphi consensus. Journal of Clinical Epidemiology 1998;51(12):1235-41.

***WHO 1979***
World Health Organization. Handbook for Reporting Results of Cancer Treatment. Geneva: World Health Organization, 1979.

***Zhang 2003***
Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. New England Journal of Medicine 2003;348(3):203-13.

# Chapter 7

## Immunization with a p53 synthetic long peptide vaccine induces p53-specific immune responses in patients with ovarian cancer, a phase II trial



Leffers N*, Lambeck AJA*, Gooden MJM, Hoogeboom BN, Wolf R, Hamming LE, Hepkema BG, Willemse PHB, Molmans BHW, Hollema H, Drijfhout JW, Sluiter WJ, Valentijn ARPM, Fathers LM, Oostendorp J, van der Zee AGJ, Melief CJ, van der Burg SH, Daemen T, Nijman HW

* Both authors contributed equally

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## ABSTRACT

The prognosis of ovarian cancer, the primary cause of death from gynaecological malignancies, has only modestly improved over the last decades. Immunotherapy is one of the new treatment modalities explored for this disease. To investigate safety, tolerability, immunogenicity and obtain an impression of clinical activity of a p53 synthetic long peptide (p53-SLP) vaccine, twenty patients with recurrent elevation of CA-125 were included, eighteen of whom were immunized four times with ten overlapping p53-SLP in Montanide ISA51. The first five patients were extensively monitored for toxicity, but showed no ≥ grade 3 toxicity, thus accrual was continued. Overall, toxicity was limited to grade 1 and 2, mostly loco-regional, inflammatory reactions. IFN-γ producing p53-specific T-cell responses were induced in all patients who received all four immunizations as measured by IFN-γ ELISPOT. An IFN-γ secretion assay showed that vaccine-induced p53-specific T-cells were $CD4^+$, which produced both Th1 and Th2 cytokines as analyzed by cytokine bead array. Notably, Th2 cytokines dominated the p53-specific response. P53-specific T-cells were present in a biopsy of the last immunization site of at least 9/17 (53%) patients, reflecting the migratory capacity of p53-specific T-cells. As best clinical response, stable disease, evaluated by CA-125 levels and CT-scans, was observed in 2/20 (10%) patients, but no relationship was found with vaccine-induced immunity. This study shows that the p53-SLP vaccine is safe, well tolerated and induces p53-specific T-cell responses in ovarian cancer patients. Upcoming trials will focus on improving T helper-1 polarization and clinical efficacy.

## INTRODUCTION

Ovarian cancer is the most frequent cause of death from gynaecological malignancies. As a result of the absence of specific symptoms, the majority of patients present with advanced stage disease. Despite standard treatment almost all patients will relapse, with a median progression-free survival of only 18 months. Notwithstanding advances in chemotherapeutic strategies in the last decades, five-year survival remains low at ~40% (1;2). Immunotherapy is considered to be a promising potential novel therapeutic strategy to treat ovarian cancer as a far more favourable prognosis is observed for those of whom the tumour is strongly infiltrated by T-cells (3;4).

One of the possible targets for intra-tumoural T-lymphocytes identified in ovarian cancer is the tumour-suppressor protein p53. This protein is activated upon DNA damage and arrests the cell cycle to allow for DNA repair or apoptotic cell death. Overexpression of the p53 protein is observed in 50-60% of ovarian cancers (5;6) and is associated with mutations of the p53 gene in 50% of these cases (7). Because of altered processing and expression of p53 in tumour cells when compared with normal cells, p53 could serve as a target tumour antigen for the immune system (8).
A large body of evidence shows that p53 can function as a target for both the humoral and cellular arm of the immune system in cancer patients. P53-specific serum auto-antibodies (p53-Aab) can be detected in patients with many types of cancer (9) and in ~18-25% of ovarian cancer patients, indicating the presence of p53-specific T-helper (Th) cells (10-14). Indeed, circulating and tumour-infiltrating p53-specific memory T-cells could be detected and isolated from patients with ovarian cancer but not benign ovarian tumours (15). In addition, p53-specific memory T cells have been observed in patients with colorectal cancer (15-17) and breast cancer (18), indicating that tumour-derived p53 can effectively be presented to the immune system and activate circulating p53-specific T-lymphocytes. Although accumulating evidence suggests that the p53-specific $CD8^+$ T-cell repertoire is restricted by self tolerance (19;20), the $CD4^+$ T-cell repertoire is not affected (21). P53-specific $CD4^+$ T cells by themselves are expected to play an important role in combating cancer because IFN-γ secreting $CD4^+$ Th1-cells are key in orchestrating and sustaining the local immune attack by $CD8^+$ cytotoxic T-lymphocytes (CTL) and innate immune effector cells, even in the case of MHC class II–negative cancers (22-24). Indeed, infusion of p53-specific $CD4^+$ Th-cells supported the immune response against p53 overexpressing tumours in a murine tumour model (21;25). Moreover, Th1-cells can activate dendritic cells (DC) that have ingested tumour material, allowing DC to launch an effective CTL response against the unique tumour antigens that are present in tumour cells (26). In view of the fact that p53 is overexpressed in many types of tumours, p53-specific $CD4^+$ T cells may act as 'universal' T-helper cells for

cancer and act analogous to the observed clinical remission and immunological responses to antigens other than NY-ESO-1 after the infusion of NY-ESO-1-specific T helper cells in a patient with metastatic melanoma (27). Our analyses of the p53-specific $CD4^+$ Th-cell repertoire in patients with cancer showed that in general these responses were weak (15;16) and failed to produce any of the Th1 or Th2 key cytokines (17) or were polarized towards a non-effective Th2 response (15), suggesting that the induction of a strong p53-specific Th1 response through immunization may enhance the efficacy of the anti-tumour response.

In our current study we used overlapping synthetic long peptides (SLP), constituting the most immunogenic part of the p53 protein (15-17), to enhance the p53-specific immune response in patients with cancer. Because of their length, SLP are predominantly taken up by professional antigen presenting cells (APC) where they are processed for presentation by both MHC class I and II molecules (28). Mixes of SLP are likely to contain multiple HLA class I and II T-cell epitopes which allows the use of this type of peptide vaccines in all patients irrespective of the type of HLA of each patient (29). The efficacy of SLP vaccines to induce effective $CD4^+$ and $CD8^+$ T-cell responses was demonstrated in rodents (30) and patients with cervical cancer (31;32). In parallel, injection of p53-SLP resulted in a strong p53-specific $CD4^+$ T-cell response to several different epitopes in mice (21).

To evaluate the safety and immunogenicity of the p53-SLP vaccine in patients with ovarian cancer, we performed a phase II clinical trial in patients with recurrent disease after primary standard treatment. Evaluation of clinical responses to the p53-SLP vaccine was a secondary objective of this study.



**Table 1** Position and sequence of amino acids used for p53 synthetic long peptides

| Vaccine peptide | Position | Amino acid sequence |
|---|---|---|
| 1 | 70-99 | APPVAPAPAAPTPAAPAPAPSWPLSSSVPS |
| 2 | 86-115 | APAPSWPLSSSVPSQKTYQGSYGFRLGFLH |
| 3 | 102-131 | TYQGSYGFRLGFLHSGTAKSVTCTYSPALN |
| 4 | 126-155 | YSPALNKMFCQLAKTCPVQLWVDSTPPPGT |
| 5 | 142-171 | PVQLWVDSTPPPGTRVRAMAIYKQSQHMTE |
| 6 | 157-186 | VRAMAIYKQSQHMTEVVRRCPHHERCSDSD |
| 7 | 174-203 | RRCPHHERCSDSDGLAPPQHLIRVEGNLRV |
| 8 | 190-219 | PPQHLIRVEGNLRVEYLDDRNTFRHSVVVP |
| 9 | 206-235 | LDDRNTFRHSVVVPYEPPEVGSDCTTIHYN |
| 10 | 224-248 | EVGSDCTTIHYNYMCNSSCMGGMNR |

## MATERIAL AND METHODS

### Participants

Adult patients with epithelial ovarian cancer and rising CA-125 level after prior systemic treatment (terminated ≥4 weeks) who were not (yet) eligible for renewed chemotherapy were included. Additional inclusion criteria were WHO performance status 0-2, life expectancy >3 months, and adequate bone marrow function. Exclusion criteria were high-dose immunosuppressive medication, symptoms consistent with CNS metastases, secondary malignancies, and severe cardiac, neurological or psychiatric co-morbidity.

### Vaccine and treatment scheme

The p53-SLP vaccine consisted of 10 synthetic 25-30 amino acids long overlapping peptides, spanning amino acids 70-248 of the wt-p53 protein (Table 1; patent number WO2008147186). Peptides were prepared at the GMP facility of the Department of Clinical Pharmacy & Toxicology of the LUMC (31;32). At the day of immunization, peptides (0.3 mg / peptide) were dissolved in dimethyl sulfoxide (DMSO, final concentration 20%) admixed with 20 mM phosphate buffer (pH 7.5) and emulsified with an equal volume of Montanide ISA51. The vaccine was administered subcutaneously 4 times with a 3-week interval. Dose and route of immunizations were chosen based on results from HPV16 E6/E7 synthetic long peptides trials (31;32). To ascertain immunizations did not result in severe toxicity, the first five patients were extensively monitored up to 3 hr after each immunization. In the absence of ≥ CTC grade 3 toxicity after a minimum of 2 immunizations in these 5 patients, inclusions would continue. Study treatment was stopped in case of progressive disease necessitating other forms of anti-tumour therapy or persisting severe toxicity. Immunizations were administered between July 2006 and August 2007.

### Clinical monitoring

Toxicity was evaluated in patients who received ≥1 immunization and graded according to Common Terminology Criteria for Adverse Events v3.0 (CTC). Relationship to treatment was evaluated for all adverse events. At each visit, patients were assessed by physical examination, vital signs, complete blood count with differential and serum biochemistry. Anti-nuclear antibodies (ANA) were evaluated before the first and after the last immunization. Patients were asked to keep a diary to record temperature, adverse events and concomitant medication. Tumour response to treatment, a secondary endpoint, was evaluated according to GCIG criteria (33) by combining serum CA-125 levels obtained at every visit with computerized tomography performed 6-9 weeks after the last immunization and evaluated according to RECIST criteria (34).

**Immunomonitoring**

***Lymphocytes and sera***

Blood for immunological assays was obtained at each visit. Immunogenicity was evaluated in patients with ≥ 1 post-immunization sample. Sera were isolated from clotted blood and cryo-preserved. PBMC isolated from heparinised blood by Ficoll-Paque density centrifugation were frozen until use in liquid nitrogen. A 6 mm skin biopsy was obtained from the last immunization site 3 weeks after the last immunization to determine presence of p53-specific T-cells. Biopsy tissue was manually cut into small pieces and the infiltrating lymphocytes were expanded by homeostatic proliferation as reported previously (15;35) and according to standard operation procedure. Briefly, to stimulate homeostatic proliferation tissue was suspended in 4ml of medium mix containing 10% autologous serum, 5% TCGF, 4µl IL-15 (5ng/ml), 8µl Gentamicine (20µg/ml) and 4µl IL-7 (5ng/ml) and distributed into four wells of a 48-wells plate. Depending on cell growth, samples were split into more wells and medium mix was added until enough cells were available (2-3 weeks of culture) for the detection of p53-specific T-cells by proliferation. In the absence of sufficient visible growth within the first 4 days, feeder cells (irradiated autologous PMBC) were added on day 4. Twice weekly culture medium was refreshed with new medium mix (without IL-7).

***Antigens used in immunological assays***

The different GMP-grade p53-SLP peptides were divided into 4 pools: p1-2 (aa 70-115), p3-4 (aa 102-155), p5-7 (aa 142-203) and p8-10 (aa 190-248). Memory recall mix (MRM), a mixture of tetanus toxoid (150 limus flocculentius/mL; RIVM, Bilthoven, the Netherlands), *M. tuberculosis* sonicate (2.5 µg/mL; generously donated by Dr P Klatser, KIT, Netherlands) and *C. albicans* (0,005% HAL Allergenen Lab, Haarlem, the Netherlands) was used to control the capacity of PBMC to proliferate in response to typical recall antigens (36).

***IFN-γ ELISPOT assay***

The IFN-γ ELISPOT assay optimized to measure p53-specific T-cell responses was performed according to standard operation procedures as described previously (15;32;36). Briefly, 1-2 million PBMC were stimulated with the indicated vaccine peptide pools (10 µg/peptide/mL) or MRM (1:50) and cultured in Iscove's medium (Gibco) containing 10% human AB serum (sigma), penicillin, streptomycin and β-mercapto-ethanol for 4 days. Then cells were counted and seeded in quadruplicates in an IFN-γ ELISPOT plate at 100,000 cells per well. After 16 hr, the plates were developed and analyzed by ELI.SCAN ELISPOT scanner (AELVIS GmbH, Hannover, Germany). A response was considered p53-specific if [(mean number of spots in experimental wells) – (mean number of spots in medium + 2 x SD)] ≥ 10

spots / $10^5$ PBMC. A vaccine-induced response was defined as a p53-specific response which exceeded the pre-existing immune response at least three-fold (32).

***Proliferation assays***

Freshly isolated PBMC were stimulated with vaccine and non-vaccine peptide pools (10 µg/peptide/mL) or MRM (1:156) in a 6-day lymphocyte stimulation test (LST) according to standard operation procedures as previously described (32). Skin-biopsy derived lymphocytes were analyzed for the presence of p53-specific T cells by stimulation with autologous monocytes pulsed overnight with the indicated vaccine peptides in a standard 3 day proliferation assay (35). Supernatants, isolated at day 5 (PBMC) or day 2 (skin biopsy derived lymphocytes) of the proliferation assay were cryo-preserved. A p53-specific response was defined as $^3$H-thymidine incorporation >1000 counts/min and a stimulation index ≥ 3 (SI = mean of p53-induced proliferation / mean of medium control) (15). A vaccine-induced response was defined as a p53-specific response with proliferation after four immunizations / proliferation before immunization ≥ 2.

***IFN-γ secretion assay***

To analyze if p53-specific responses were CD4 or CD8 mediated, PBMC were stimulated for 4 days with the vaccine peptides or medium as described above for the ELISPOT assay and analyzed by IFN-γ secretion assay (Miltenyi Biotec, Utrecht, The Netherlands) according to manufacturer's instructions. Briefly, stimulated PBMC were harvested, washed with ice-cold buffer (PBS/BSA 0.5%/EDTA 2 mM) and incubated in 90 µl cold medium (per $10^6$ cells) containing anti-IFN-γ antibody conjugated to cell surface specific monoclonal antibodies (10 µl/$10^6$ cells) for 5 min on ice. Next, PBMC were 10x diluted in warm medium and incubated for 45 min at 37°C under slow continuous rotation. After incubation, PBMC were washed and incubated in 90 µl ice-cold buffer containing 10 µl anti-IFN-γ (per $10^6$ cells), anti-CD4 APC (IQproducts, Groningen, the Netherlands) and anti-CD8 PECy5 (IQ Products, Groningen, the Netherlands). After 10 min incubation on ice, PBMC were washed, suspended in 300 µl ice-cold buffer, and analyzed by flow cytometry for the presence of $CD4^+IFN\text{-}\gamma^+$ or $CD8^+IFN\text{-}\gamma^+$ T cells.

IFN-γ production by $CD4^+$ or $CD8^+$ T-cells was considered to be p53-specific if after stimulation with vaccine peptides the percentage of IFN-γ producing $CD4^+$ or $CD8^+$ T-cells within the $CD4^+$ or $CD8^+$ T-cell population was at least twice as high as the percentage of non-stimulated (medium only) IFN-γ producing T-cells. A vaccine-induced response was defined as a p53-specific response after immunization that was ≥ 3 fold higher than the percentage of $IFN\text{-}\gamma^+$ T-cells in the pre-immunization sample.

***Cytokine bead array***

Production of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α was evaluated in supernatants of proliferation assays by cytokine bead array (LINCO*plex kit*, Linco Research, St. Charles, MO) as described earlier (15). P53-specific cytokine production was defined as concentration of cytokine ≥ 2 medium control and if concentration ≥ 100 pg/ml (IFN-γ), or ≥ 10 pg/ml (other cytokines). A vaccine-induced response was defined as a p53-specific post-immunization concentration ≥ 2 pre-immunization concentration (PBMC only).

***Immunohistochemistry***

Primary tumours were evaluated for p53 expression (DO-7, 1:1000 in PBS 1% BSA, DAKO) (15). Tumours with > 50% moderate or strong immunostaining were considered to have p53-overexpression (6;37).

***Flow cytometry***

PBMC were evaluated for CD3, CD4, CD8, CD19, CD56 (IQ Products, Groningen, the Netherlands) and FoxP3 (eBioscience, San Diego, CA) by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium) according to manufacturer's instructions. Skin biopsy derived lymphocytes were analyzed for CD3, CD4, CD8, and if possible Foxp3.

**Ethics statement**

The study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen and conducted in adherence with the principles of the Declaration of Helsinki. All patients gave written informed consent. An independent agency (Trial Coordination Center, Groningen, The Netherlands) was contracted to monitor the study and adherence to GCP principles.



**Statistical analysis**

It was calculated that to ascertain a 95% probability of an immunologic response rate of at least 15%, minimally 19 patients would have to be immunized. Differences between pre- and post-immunization were evaluated using a t-test for paired comparisons or a Wilcoxon's signed-ranks test. Differences in response at different time points for normally distributed quantitative variables were evaluated by repeated measures analysis with linear mixed modelling, or Mantel-Haenzel (MH) statistics. For qualitative variables, overall differences were evaluated using McNemar's test or MH-strategy. MH-statistics were used to determine if average responses differed at the different time points ($Q_{SMH}$). Statistical significance was defined as $P < .05$. Statistical Analysis System version 9.1 (SAS Institute, Cary, NC) was used for all analyses.

## RESULTS

### P53-SLP vaccine is safe and well tolerated

Informed consent was obtained from 23 patients, but three patients failed screening (P07, P10 & P16). Because of rapidly progressive disease, two patients received two immunizations only (P04 & P12). All other patients (N = 18) received four immunizations. P53-overexpression in the primary tumour was demonstrated by immunohistochemical analysis in 50% (10/20) of patients (Table 2). As no vaccine-related CTC grade 3 or 4 adverse events were observed after a minimum of 2 immunizations in the first 5 patients, it was considered safe to continue inclusions. Similarly, no severe vaccine-related adverse events occurred in the subsequently included and immunized patients (Table 3). No major vaccine-induced alterations were observed in serum biochemistry and complete blood count. Mild to moderate inflammatory symptoms at the injection site occurred in nearly all patients; redness and swelling lasted 7-8 days on average, pain and itch 3-4 days on average. Activation of local inflammatory symptoms at prior injection sites was seen in 60% of patients after subsequent immunizations. We did not find evidence of vaccine-induced auto-immunity, assessed by clinical symptoms and levels of serum ANA levels (data not shown). P53-autoantibodies, present in 40% (8/20) of the patients before immunization, were associated with p53-overexpression in the primary tumour ($P = 0.023$; Fischer's exact test). After 1 or more immunizations, p53-autoantibodies were present in 45% (9/20) of patients. Flow-cytometric analysis of the major lymphocyte subpopulations (T-cells, B-cells, NK-cells) revealed that these remained relatively stable during immunization albeit that the percentage of $CD3^+$ T-cells was somewhat lower after 2 (mean ± SEM: 63.2 ± 3.4) and 3 immunizations (65.9 ± 2.4) compared to before immunization (70.4 ± 1.7). Within the CD3+ T-cell population the percentages of $CD4^+$, $CD8^+$ T-cells, and $CD4^+FoxP3^+$ T-cells remained constant at 40%, 25% and 7%, respectively (data not shown).

### IFN-γ producing T-cells are induced by the p53-SLP vaccine in all patients

We analyzed p53-specific T-cell responses after every consecutive immunization by IFN-γ ELISPOT. Before immunization, responses against peptides included in the vaccine were present in patients P11 (all vaccine peptide pools), P12 (vaccine peptides 3-4) and P19 (vaccine peptides 1-2, 3-4 and 5-7). In all patients who completed the immunization scheme (N = 18), vaccine-induced IFN-γ producing p53-specific T-cells could be detected at two or more time points (Table 4). A number, but not all, of the pre-existing p53-specific responses were boosted (Table 4). The strength of the vaccine-induced p53-specific T-cell response was at its peak in the circulation after one immunization (median number of cumulative spots against all vaccine peptide pools 276, interquartile range (IQR) 91-400) after which the number

of circulating IFN-γ producing p53-specific T-cells decreased after each subsequent immunization (median (IQR): 214 (63-266), 169.0 (65-188), 93 (28-122) respectively after 2, 3 and 4 immunizations). The responsiveness to the mix of recall antigens (MRM) remained stable over time ($P = .625$; Wilcoxon's signed rank test), indicating that the change in p53-reactivity was the result of p53-specific immunization.

**The p53-SLP vaccine induces proliferating p53-specific T-cells**

In addition, the capacity of p53-specific T-cells to proliferate upon antigenic stimulation was analyzed before and after 4 immunizations. Pre-existing p53-SLP vaccine-specific proliferative responses were detected in patients P18 (vaccine peptides 5-7, SI 4.4) and P19 (vaccine peptides 1-2 (SI 3.5), 5-7 (SI 5.9) and 8-10 (SI 3.7)). After 4 immunizations, vaccine-induced p53-specific responses against the vaccine peptides were observed in 82.4% (14/17) of patients (Fig 1b). The pre-existing p53-specific proliferative responses present in P18 and P19 were not significantly boosted by immunization. Similar to the results of the IFN-γ ELISPOT the p53-specific proliferative responses against all, but vaccine peptides 1-2, were higher after four immunizations than before immunization ($P < .02$; Wilcoxon's signed rank test). Furthermore, all patients except for 2 (P2 & P17) responded to at least 2 different peptide pools. No differences were detected in the proliferative response to MRM ($P = .927$; Wilcoxon's signed rank test)).

**Differences in immunogenicity and magnitude of responses to p53-SLP**

To make a distinction in the immunogenicity of the injected p53-peptides we compared the strength of the p53-specific T-cell response to each of the 4 different peptide pools (vaccine peptides 1-2, 3-4, 5-7, and 8-10) as measured by IFN-γ ELISPOT (Fig. 1a). The strength of the responses against p53 vaccine peptides 3-4, 5-7 and 8-10 was at all time points higher than responses against vaccine peptides 1-2 ($P < .01$; Wilcoxon's signed rank test), suggesting that the immunogenicity of the first pool of peptides is low within this patient population. After 1, 2 and 3 immunizations, responses against p53 vaccine peptides 8-10 were also higher than responses against vaccine peptides 3-4 ($P < .02$; Wilcoxon's signed rank test). Similar differences were observed in the strength of proliferation induced by the 4 different pools of peptides (Fig. 1b).

**Vaccine-induced p53-specific T-cell immunity is mediated predominantly by $CD4^+$ type 2 T-cells.**

To analyze if the vaccine-induced p53-specific T-cell responses were CD4- or CD8-mediated PBMC of 5 patients (P01, P08, P21, P22, P23), selected on basis of their strong reactivity in the IFN-γ ELISPOT assay, were stimulated for 4 days with the pools of vaccine peptides that induced high IFN-γ responses in the ELISPOT and

**Table 2** Patient Characteristics

| | Age | FIGO stage | Histology | Grade | Residual disease >2cm* | Prior chemo-therapy | CT scan at inclusion[ξ] | p53 over-expression in primary tumour [a] | HLA-DR genotype |
|---|---|---|---|---|---|---|---|---|---|
| P01 | 43 | IIIc | Mucinous | 3 | No | Second line | ED | + | DRB1*01 |
| P02 | 50 | IIIc | Clear cell | 3 | Yes | Second line | ED | + | DRB1*13,*15;DRB3; DRB5 |
| P03 | 46 | IIIc | Serous | 3 | Yes | First line | ED | - | DRB1*01,*03;DRB3 |
| P04 | 50 | IIIc | Endometrioid | 3 | No | Second line | ED | - | DRB1*03,*07;DRB3;DRB4 |
| P05 | 47 | IV | Serous | 3 | Yes | First line | NED | + | DRB1*04,*15;DRB4;DRB5 |
| P06 | 68 | IIc | Endometrioid | 3 | Unknown | First line | ED | + | DRB1*13,*14;DRB3 |
| P08 | 51 | IIIc | Serous | 2 | No | Second line | NED | - | DRB1*03,*11;DRB3 |
| P09 | 66 | IIIc | Clear cell | 3 | Yes | First line | ED | + | DRB1*01,*13;DRB3 |
| P11 | 47 | IIIc | Serous | 1 | No | First line | ED | - | DRB1*10,*15;DRB5 |
| P12 | 44 | Ic | Clear cell | 2 | No | Second line | ED | - | not evaluated |
| P13 | 69 | III | Serous | 3 | Yes | Second line | ED | - | DRB1*08,*13;DRB3 |
| P14 | 67 | IIIb | Serous | 3 | No | Fourth line | ED | - | DRB1*01,*04;DRB4 |
| P15 | 56 | IIIb | Serous | 2 | No | First line | NED | + | DRB1*04,*15;DRB4; DRB5 |
| P17 | 43 | IIIc | Endometrioid | 3 | Yes | First line | NED | - | DRB1*04,*07;DRB4 |
| P18 | 50 | IIIc | Serous | 2 | Unknown | First line | ED | + | DRB1*04,*11; DRB3; DRB4 |
| P19 | 55 | IIIc | Serous | 3 | Unknown | First line | NED | + | DRB1*11,*13;DRB3 |
| P20 | 51 | IV | Serous | 3 | No | Second line | ED | + | DRB1*03,*04; DRB3; DRB4 |
| P21 | 58 | IIIc | Serous | 1 | No | First line | ED | - | DRB1*01,*11;DRB3 |
| P22 | 48 | IIIc | Serous | 2 | No | First line | ED | - | DRB1*03,*13; DRB3 |
| P23 | 52 | IIIc | Serous | 3 | No | First line | ED | - | DRB1*11,*11; DRB3 |

FIGO = International Federation of Gynecology and Obstetrics; * residual disease after primary surgery; [ξ] ED = evidence of disease, NED = no evidence of disease. [a] P53 expression in the primary tumour analyzed by immunohistochemistry using the p53-specific antibody DO-7.

then analyzed by an IFN-γ secretion assay using flow cytometry. Similar to the results obtained by IFN-γ ELISPOT we did not observe an IFN-γ associated p53-specific response in the pre-immunization samples of these patients whereas vaccine-induced IFN-γ producing T-cell responses were detected in all patients after immunization. All the p53-specific IFN-γ responses were mediated by $CD4^+$ T-cells (Fig. 2a; Table 5). No vaccine-induced $CD8^+$ T-cell responses could be detected.

To determine the polarization of the vaccine-induced T-cell response, the p53-specific production of both Th1 (IL-2, IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5, and IL-10) was measured in the supernatants isolated from the proliferation assays of a subset of 8 patients of whom both PBMC and lymphocytes cultured from skin biopsies (see below) displayed a p53-specific proliferative response (Fig. 2b)

Analyses of the culture supernatants of these proliferation assays revealed that p53-specific proliferation coincided with the production of both Th1 and Th2 cytokines. In PBMC, vaccine-induced production of both Th1 cytokines (median [range] IL-2: 19.7 [12.1-27.3] pg/ml; IFN-γ 193 [123-486] pg/ml; TNF-α 109 [41.8-1255] pg/ml) and Th2 cytokines was detected (IL-5 68.6 [14.4-224] pg/ml; IL-10 145 [45.8-347] pg/ml) as determined by cytokine bead array. IL-5 and IL-10 were more frequently produced than IL-2 and IFN-γ. In lymphocytes cultured from skin biopsies, also vaccine-induced

**Table 3** Number of Adverse Events and Number of Patients with Adverse Events

| | Number of events (number of patients) | | | | |
|---|---|---|---|---|---|
| CTC grade[§] | 1 | 2 | 3 | 4 | 5 |
| ALAT | 2 (2) | - | - | - | - |
| ASAT | 3 (3) | - | - | - | - |
| LDH | 4 (4) | - | - | - | - |
| Anaemia | 5 (5) | - | - | - | - |
| Leucocytes | 8 (8) | 1 (1) | - | - | - |
| Platelets | 5 (5) | - | - | - | - |
| Hematoma (not injection site) | 2 (2) | 1 (1) | - | - | - |
| Cardiovascular | 8 (5) | 1 (1) | - | - | - |
| Thrombosis/Embolism | - | - | - | 1(1)* | - |
| Peripheral Edema | 2 (2) | 1 (1) | - | - | - |
| Pulmonary | 1 (1) | 1 (1) | - | - | - |
| Dyspnoea | 4 (3) | - | - | - | - |
| Constitutional symptoms | 12 (8) | 4 (3) | - | - | - |
| Fatigue | 2 (2) | 3 (1) | - | - | - |
| Diarrhoea | 1 (1) | - | - | - | - |
| Nausea | 3 (3) | - | - | - | - |
| Vomiting | 1 (1) | - | - | - | - |
| Musculoskeletal pain | 8 (6) | 9 (5) | - | - | - |
| Abdominal pain | 9 (7) | 6 (3) | 1 (1)* | - | - |
| Dermatology | 8 (6) | - | - | - | - |
| Hernia Cicatricialis | - | 2 (2) | - | - | - |
| Neurology | 4 (3) | 1 (1) | - | - | - |
| Headache | 18 (6) | 1 (1) | - | - | - |
| Anxiety | 2 (1) | - | - | - | - |
| Conjunctivitis | 1 (1) | 1 (1) | - | - | - |

[§]adverse events are graded according to CTC criteria v3.0; * related to disease progression

**Table 4.** Vaccine-Induced P53-specific Immune Responses in PBMC of Ovarian Cancer Patients Immunized with the P53-SLP Vaccine as Analyzed by IFN-γ ELISPOT

| | After one vaccination (I) | | | | After two vaccinations (II) | | | | After three vaccinations (III) | | | | After four vaccinations (IV) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient[1] | vac p1-p2[2] | vac p3-p4 | vac p5-p7 | vac p8-p10 | vac p1-p2 | vac p3-p4 | vac p5-p7 | vac p8-p10 | vac p1-p2 | vac p3-p4 | vac p5-p7 | vac p8-p10 | vac p1-p2 | vac p3-p4 | vac p5-p7 | vac p8-p10 |
| P01 | - | 623 | 17 | 106 | - | 14 | - | 33 | - | - | - | - | - | 20 | - | 56 |
| P02 | - | - | - | - | - | - | - | 28 | - | - | - | 21 | - | - | 66 | 27 |
| P03 | - | 52 | 37 | 166 | - | 29 | 15 | 81 | - | 43 | 58 | 132 | - | 15 | 20 | 57 |
| P04 | - | - | - | - | - | - | - | - | na | na | na | na | na | na | na | na |
| P05 | - | 19 | 19 | 11 | - | - | - | - | - | - | 29 | 18 | - | - | - | - |
| P06 | - | 12 | 47 | 22 | - | - | - | 21 | - | - | - | - | - | - | - | - |
| P08 | 110 | 131 | 241 | 220 | 76 | 130 | 200 | 303 | 42 | 113 | 44 | 219 | - | 59 | 32 | 156 |
| P09 | - | 31 | 38 | 55 | - | 10 | 18 | 20 | - | 14 | 12 | - | - | - | - | - |
| P11 | - | 71 | 120 | 138 | - | - | 78 | - | - | - | 35 | - | - | - | 28 | - |
| P12 | 26 | - | 67 | 73 | na | na | na | na | na | na | na | na | na | na | na | na |
| P13 | - | 65 | 65 | 81 | 37 | 83 | 133 | 144 | 16 | - | 125 | 93 | - | - | 49 | 21 |
| P14 | - | 60 | - | 22 | - | - | 12 | 48 | - | - | - | - | - | - | - | - |
| P15 | - | - | 18 | - | - | 22 | 20 | - | - | - | 14 | - | - | - | - | - |
| P17 | - | 63 | 46 | 21 | - | - | 52 | 20 | - | - | - | - | - | - | - | - |
| P18 | - | 88 | 106 | 120 | - | 41 | 61 | 45 | - | - | - | 60 | - | 41 | 13 | - |
| P19 | - | - | 183 | 94 | - | - | 75 | 60 | - | - | - | - | - | - | - | 23 |
| P20 | - | - | - | 34 | - | 11 | - | 28 | - | 10 | - | 53 | - | - | - | - |
| P21 | 22 | 119 | 100 | 178 | - | 88 | 81 | 32 | - | 33 | 38 | 32 | - | 12 | 16 | - |
| P22 | 14 | 80 | 257 | 367 | 21 | 57 | 201 | 295 | - | 77 | 217 | 345 | - | - | - | 64 |
| P23 | - | 50 | 106 | 21 | - | - | 35 | - | - | 20 | 55 | 22 | - | 13 | - | - |

[1]Patients analyzed for p53-specific responses before and after every immunization (time points I-IV) by IFN-γ ELISPOT. [2]The pool of p53 vaccine peptides used to stimulate patient-derived PBMC *in vitro* for 4 days. [3]Only vaccine-induced p53-specific responses are shown (see definition in Material and Methods). Responses are depicted as number of specific spots per $10^5$ PBMC (mean of experimental wells – (mean + 2xSD) of medium control). Responses to medium stimulated wells were low (median 3.75, IQR 1.5-8.75 spots per $10^5$ PBMC). Pre-existing responses were present in P11 against p1-2, p3-4, p5-7, p8-10 (19, 12, 11 and 30 spots respectively); P12 against p3-4 (25 spots); and P19 against p1-2, p3-4, p5-7 (19, 19 and 11 spots respectively). - = no vaccine-induced p53-specific response; na = PBMC were not available.

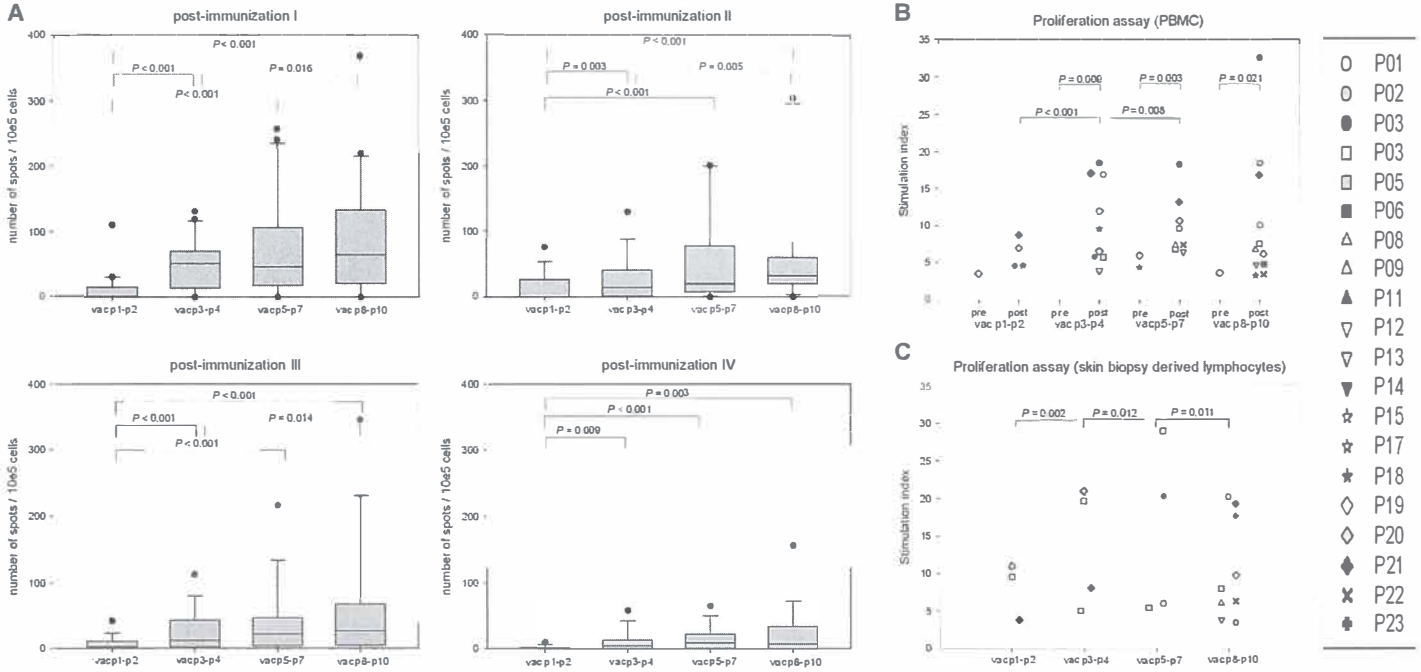



**Fig 1.** P53-specific responses in ovarian cancer patients immunized with the p53-SLP vaccine. (A) Boxplots comparing responses to vaccine peptide pools as analyzed by IFN-γ ELISPOT using PBMC after I-IV immunizations. The number of p53-specific IFN-γ producing cells (per $10^5$ PBMC) was calculated by subtracting the mean number of spots+2xSD of the medium from the mean number of spots of the experimental wells (vertical axis). (B/C) P-53 specific responses to vaccine peptides as measured by proliferation assay using PBMC obtained before immunization and after four immunizations (B) or skin biopsy derived lymphocytes from the last injection site (C).The vertical axis represents the stimulation index calculated as the mean $^3$H-thymidine incorporation of the experimental wells divided by the mean of the medium control.

production of both Th1 cytokines (IL-2: 275 [28.3-708] pg/ml; IFN-γ 262 [111-461] pg/ml; TNF-α 426 [88.0-676] pg/ml) and Th2 cytokines (IL-4: 653 [45.4-22924] pg/ml; IL-5 1606 [35.7-10242] pg/ml; IL-10 1167 [62.0-3555] pg/ml) was found. Based on predefined cut-offs, all analyzed patients showed a predominance in the p53-specific Th2 response, as the frequency of Th2 responses and the amounts of Th2 cytokines produced are higher than Th1 cytokines (Fig. 2b).

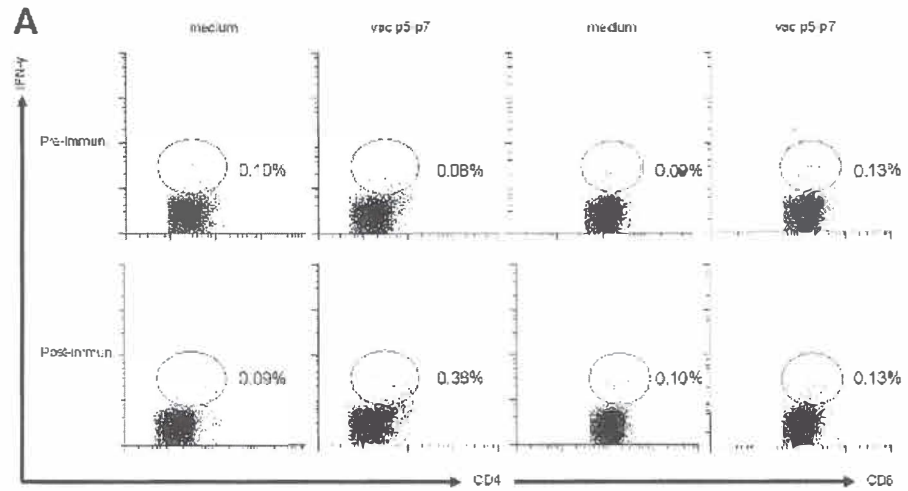
*Vaccine-induced p53-specific T-cells migrate into the immunization sites.*

To analyze the capacity of p53-specific T-cells to migrate to sites where p53 antigen is presented, we cultured lymphocytes from skin biopsies taken at the fourth injection site (n=17) using a successful protocol to obtain antigen-specific T-cells, which is based on the induction of homeostatic proliferation and as such does not strongly affect the phenotype of the infiltrating lymphocytes, as published previously (15;35;38). Only in 2 cases (P15 and P20) we were not able to obtain sufficient numbers of lymphocytes from the skin biopsy to analyze the p53-specific T-cell response by proliferation assay. The median yield of cells was 1.25 x $10^6$ (range 0.06 x $10^6$ – 11.1 x $10^6$) after 2-3 weeks of culture. Flow cytometric-assisted phenotyping of the infiltrating lymphocytes showed a preponderance of $CD3^+$ T-cells in skin biopsy derived lymphocytes (mean ± SEM: 72.5% ± 7.8), of which 58.4% ± 7.2 were $CD4^+$, 12.6% ± 2.4 were $CD8^+$ and the percentage of $CD4^+Foxp3^+$ cells was 2.7% ± 0.7. Vaccine-site infiltrating p53-specific T-cells were detected in 52.9% (9/17) of the tested patients (Fig. 1c). The p53-specific responses in skin-derived lymphocytes and PBMC were not always directed against the same epitopes (Fig. 2b).

**Tumour responses**

Two patients received only 2 immunizations due to rapidly progressive disease. All other patients were evaluated for tumour response after the fourth immunization. Two patients (10%) had stable disease as evaluated by CA-125 and computerized tomography (P17, P23). In both patients vaccine-induced p53-specific responses were present. The remainder of patients (18/20; 90%) had clinical, biochemical and/or radiographic evidence of progressive disease.

**Fig 2.** Phenotyping of p53-specific responses induced by the P53-SLP vaccine. (A) An illustrative example (P23) of induction of IFN-γ secreting $CD4^+$ T-cells, but not $CD8^+$ T-cells after immunization as analyzed by IFN-γ secretion assay. (B) Th1/Th2 cytokine production in PBMC and skin biopsy derived lymphocytes after four immunizations with the p53-SLP vaccine. Cytokines were measured in supernatants of cultures with a vaccine-induced p53-specific (PBMC) or p53-specific response (skin biopsies) as measured by proliferation assay. Vaccine-induced p53-specific responses (PBMC) or p53-specific responses (skin biopsies) in proliferation assays are represented by a box. Vaccine-induced increase in production of a cytokine (PBMC) or positive production of a cytokine (skin biopsy) is indicated by a filling (white: <2x cut off value; yellow: 2-5x cut-off; orange: 5-15x cut-off; red: 15-50x cut-off; and dark red: >50x higher than cut-off).




B

| Patient | | vac p1-p2 | vac p3-p4 | vac p5-p7 | vac p8-p10 |
|---|---|---|---|---|---|
| P01 | PBMC | | | | |
| | Skin biopsy | | | | |
| P02 | PBMC | | | | |
| | Skin biopsy | | | | |
| P05 | PBMC | | | | |
| | Skin biopsy | | | | |
| P08 | PBMC | | | | |
| | Skin biopsy | | | | |
| P19 | PBMC | | | | |
| | Skin biopsy | | | | |
| P21 | PBMC | | | | |
| | Skin biopsy | | | | |
| P22 | PBMC | | | | |
| | Skin biopsy | | | | |
| P23 | PBMC | | | | |
| | Skin biopsy | | | | |

| | | |
|---|---|---|
| IL-2 | IFN-γ | TNF-α |
| IL-4 | IL-5 | IL-10 |

## DISCUSSION

This phase II clinical trial shows that a synthetic p53 long peptide (p53-SLP®) vaccine, constituting the middle part of the p53 protein, emulsified with Montanide ISA51 is safe, well-tolerated and capable of inducing p53-specific $CD4^+$ T-cell responses in 90% of ovarian cancer patients as detected on at least two different time points. The number of circulating IFN-γ producing p53-specific T-cells peaked after the first stimulation and then subsided, but was always higher than before immunization (Fig. 1). A similar decrease in vaccine-induced p53-specific T-cell reactivity after multiple immunizations has also been observed in patients with breast cancer who were vaccinated with p53-peptide pulsed dendritic cells (39). Formally, it cannot be excluded that the constant release of p53 peptides from the immunization sites may have resulted in the partial induction of hypo-responsiveness or apoptosis of p53-specific T-cells, but the isolation of poly-functional p53-specific T-cells from the immunization sites argues against this. In addition, an increase in regulatory T-cell activity due to repeated immunizations cannot be ruled out. This would imply that immunization resulted in the induction of p53-specific regulatory T-cells. Despite the fact that immunization with HPV16 peptides induced HPV16-specific regulatory T-cells in patients with cervical cancer (32), p53-specific regulatory T-cells were not detected following p53-specific immunization of patients with colorectal cancer (40). Moreover, the number of $CD4^+Foxp3^+$ (regulatory) T-cells remained constant before and after immunization (not shown). It is more likely that the decrease in the number of circulating p53-specific T-cells is due to the emigration of vaccine-activated p53-specific T-cells from the blood into the extra-lymphoid organs (41) as illustrated by the detection of p53-specific T cells in the immunization sites (Fig. 1 and 2). The p53-SLP®-vaccine induced p53-specific T-cells against multiple epitopes in the majority of patients (Table 4, Fig. 1 and 2), but responses to the first part of the vaccine (p1-p2) were infrequently observed.



**Table 5** P53-specific IFN-γ Production by $CD4^+$ and $CD8^+$ T-cells as Analyzed by IFN-γ Secretion Assay

| Patient | vac. pep. | IFN-γ producing $CD4^+$ T-cells (%) | | IFN-γ producing $CD8^+$ T-cells (%) | |
|---|---|---|---|---|---|
| | | Pre-immun. | Post-immun. | Pre-immun. | Post-immun. |
| P01 | vac p8-p10 | -0.03 | **0.36** | 0.05 | 0.10 |
| P08 | vac p5-p7 | -0.08 | 0.03 | -0.07 | -0.17 |
| | vac p8-p10 | 0.05 | **0.50** | -0.22 | -0.11 |
| P21 | vac p3-p4 | -0.01 | **0.38** | 0.15 | 0.06 |
| | vac p8-p10 | -0.01 | **0.38** | 0.00 | 0.05 |
| P22 | vac p5-p7 | -0.01 | **0.44** | -0.09 | 0.02 |
| | vac p8-p10 | 0.00 | **0.66** | -0.08 | 0.06 |
| P23 | vac p5-p7 | -0.02 | **0.29** | 0.04 | 0.03 |

The percentage of IFN-γ producing T-cells before immunization (pre-immun.) and after immunization (post-immun.) upon stimulation with the p53 vaccine peptide pools is shown. Responses are depicted as the percentage of IFN-γ production by the peptide-stimulated cells – the medium control (median 0.115, IQR 0.100-0.205). Vaccine-induced p53-specific T-cell responses are shown in bold (see definition in Material and Methods).

This is reminiscent of our prior studies examining spontaneous response to p53. In these studies 3 large pools of peptides were used and the amino acid sequence covered by p1-p2 was present in the first pool of peptides. This first pool of peptides was only sporadically recognized, whereas the second pool of p53 peptides (covering p3-p10) was predominantly recognized (15;16), suggesting that peptides 1 and 2 of the p53-SLP do not encode HLA class II epitopes for the particular types of MHC class II molecules within this group of Dutch patients. Notably, scrutiny of vaccine-induced immunity vs. HLA class II (Table 2) did not reveal any correlation between responsiveness and a particular HLA class II type indicating that the responses are likely to be restricted by multiple MHC class II molecules.

Although p53-SLP immunization resulted in the expansion of p53-specific Th1 and Th2 $CD4^+$ T-cell responses, the production of Th2 cytokines dominated both in frequency and amount (Fig. 2). Notably, Th2 cytokines were also the main product of the spontaneous immune response against p53 in patients with ovarian cancer (15). Likewise, colorectal cancer patients with tumour-induced (17) or p53-SLP vaccine-induced p53-specific T-cells (40) produced only low amounts of IFN-$\gamma$ or none of the key signature cytokines of Th1 or Th2 cells at all. Together these 2 clinical studies suggest that the current p53-SLP vaccine is capable of activating or reinforcing the same type of T-cell response as the one that spontaneously occurs in patients, albeit the vaccine-induced response is stronger and more pronounced. Based on our study in colorectal cancer we reasoned that a prolonged immunization scheme (*i.e.* multiple instead of 2 injections) may result in a stronger polarized Th1 response (40). Our current trial however, in which patients were vaccinated 4 times, reveals that this is not the case and indicates that the vaccine needs to be supplemented with strong Th1 polarizing agents. Ligands of Toll-like receptors (TLR) have been shown to act as strong Th1/CTL immunity polarizing adjuvants for vaccines (reviewed in (28;29)). A combination of such an adjuvant with the p53-SLP vaccine may result in the induction of an effective $CD4^+$ Th1 response and p53-specific CTL as well. Here, we did not detect $CD8^+$ T-cell reactivity and this was not unexpected as the p53-specific $CD8^+$ T-cell, but not the $CD4^+$ T cell repertoire is severely restricted by self tolerance and might only consist of lower affinity p53-specific $CD8^+$ T cells (19;21). However, p53-specific $CD8^+$ T-cells have been identified in cancer patients (42;43), and we can thus not rule out the possibility that with the help of an effective p53-specific Th1 response also p53-specific $CD8^+$ T-cells are activated. In favour of this are our observations that only those cervical cancer patients who were able to mount an E7-specific Th1 response upon HPV16-SLP immunization were also able to mount E7-specific $CD8^+$ T-cell immunity (32). A trial to improve Th1 polarization has been started in patients with colorectal cancer.

We observed stable disease in 2 patients with vaccine induced p53-specific T-cell responses, however, disease stabilization could not be attributed to vaccine-induced immunity and is more likely the natural course of disease in these patients. This raises the issue at which point during the course of disease immunotherapy is most effective. In ovarian cancer, clinical response rates up to 80% are obtained with first-line treatment (1) and at this point during disease pre-existing p53-specific T-cell responses are present in about half of the patients (15). However, as in the majority of patients disease will recur (44), this poses an excellent niche for immunotherapy in the adjuvant setting. Perhaps the clinical response rate would have been better when we administered the vaccine in a truly adjuvant setting, instead of in patients with recurrent, yet limited disease, who had a strikingly lower detection rate of pre-existing p53-specific T-cell immunity than expected (20%; this study). Alternatively, one could combine immunotherapy with chemotherapy. Lung cancer patients vaccinated with an adenoviral vector expressing p53 displayed better clinical responses to chemotherapy administered after immunotherapy when they had also mounted a vaccine-induced p53-specific T-cell response, hinting at a possible benefit of combining chemotherapy and immunotherapy (45). As the clinical activity of the p53-SLP vaccine can also be impeded by the incapacity of T-cells to infiltrate tumour tissue as is found in a substantial number of patients (3;46) p53-SLP immunization in combination with an endothelin B receptor (ETbR) antagonist may augment T-cell homing to tumours and reduce tumour growth similar to that found in previously ineffective immunotherapy models (46). Another problem which may inhibit anti-tumour responses and obstruct clinical efficacy are ovarian cancer infiltrating regulatory T-cells (47). Although the number of circulating $CD4^{+}FoxP3^{+}$ (regulatory) T-cells in our patient group (7.0%) as well as in another study of ovarian cancer patients (48) is relatively low and comparable to what is found in healthy subjects (48) their presence and recruitment to the tumour fosters tolerance to the tumour (47). In cancer patients, low doses of cyclophosphamide were shown to selectively deplete regulatory T-cells (49) and enhance the induction of antigen-specific T-cells as well as increase survival when combined with immunotherapy (50). We have now started a new clinical trial in which p53-SLP immunization is combined with cyclophosphamide to test whether this increases immunity and clinical activity.



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## REFERENCES


(1) Agarwal R, Linch M, Kaye SB. Novel therapeutic agents in ovarian cancer. Eur J Surg Oncol 2006;32(8):875-86.

(2) Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55(2):74-108.

(3) Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348(3):203-13.

(4) Leffers N, Gooden MJ, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, et al. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. Cancer Immunol Immunother 2009;58(3):449-59.

(5) Nijman HW, Lambeck A, van der Burg SH, van der Zee AG, Daemen T. Immunologic aspect of ovarian cancer and p53 as tumor antigen. J Transl Med 2005;3:34.

(6) de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006;95(5):627-33.

(7) Bartel F, Jung J, Bohnke A, Gradhand E, Zeng K, Thomssen C, et al. Both Germ Line and Somatic Genetics of the p53 Pathway Affect Ovarian Cancer Incidence and Survival. Clin Cancer Res 2008;14(1):89-96.

(8) Offringa R, Vierboom MP, van der Burg SH, Erdile L, Melief CJ. p53: a potential target antigen for immunotherapy of cancer. Ann N Y Acad Sci 2000 Jun;910:223-33.

(9) Soussi T. p53 Antibodies in the Sera of Patients with Various Types of Cancer: A Review. Cancer Res 2000;60(7):1777-88.

(10) Abendstein B, Marth C, Muller-Holzner E, Widschwendter M, Daxenbichler G, Zeimet AG. Clinical significance of serum and ascitic p53 autoantibodies in epithelial ovarian carcinoma. Cancer 2000;88(6):1432-7.

(11) Mayerhofer K, Tempfer C, Kucera E, Hefler L, Zeisler H, Kainz C, et al. Humoral p53 antibody response is a prognostic parameter in ovarian cancer. Anticancer Res 1999;19(1B):875-8.

(12) Gadducci A, Ferdeghini M, Buttitta F, Cosio S, Fanucchi A, Annicchiarico C, et al. Assessment of the prognostic relevance of serum anti-p53 antibodies in epithelial ovarian cancer. Gynecol Oncol 1999;72(1):76-81.

(13) Goodell V, Salazar LG, Urban N, Drescher CW, Gray H, Swensen RE, et al. Antibody immunity to the p53 oncogenic protein is a prognostic indicator in ovarian cancer. J Clin Oncol 2006;24(5):762-8.

(14) Vogl FD, Frey M, Kreienberg R, Runnebaum IB. Autoimmunity against p53 predicts invasive cancer with poor survival in patients with an ovarian mass. Br J Cancer 2000;83(10):1338-43.

(15) Lambeck A, Leffers N, Hoogeboom BN, Sluiter W, Hamming I, Klip H, et al. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007;121(3):606-14.

(16) van der Burg SH, de Cock K, Menon AG, Franken KL, Palmen M, Redeker A, et al. Long lasting p53-specific T cell memory responses in the absence of anti-p53 antibodies in patients with resected primary colorectal cancer. Eur J Immunol 2001;31(1):146-55.

(17) van der Burg SH, Menon AG, Redeker A, Franken KL, Drijfhout JW, Tollenaar RA, et al. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. Int J Cancer 2003;107(3):425-33.

(18) Tilkin AF, Lubin R, Soussi T, Lazar V, Janin N, Mathieu MC, et al. Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. Eur J Immunol 1995;25(6):1765-9.

(19) Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C, Sherman LA. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. J Exp Med 1997;185(5):833-41.

(20) Hernandez J, Lee PP, Davis MM, Sherman LA. The use of HLA A2.1/p53 peptide tetramers to visualize the impact of self tolerance on the TCR repertoire. J Immunol 2000;164(2):596-602.

(21) Lauwen MM, Zwaveling S, de Quartel L, Ferreira Mota SC, Grashorn JA, Melief CJ, et al. Self-tolerance does not restrict the CD4+ T-helper response against the p53 tumor antigen. Cancer Res 2008;68(3):893-900.

(22) Greenberg PD. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv Immunol 1991;49:281-355.

(23) Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med 1998;188(12):2357-68.

(24) Melief CJ, van der Burg SH, Toes RE, Ossendorp F, Offringa R. Effective therapeutic anticancer vaccines based on precision guiding of cytolytic T lymphocytes. Immunol Rev 2002;188:177-82.

(25) Zwaveling S, Vierboom MP, Ferreira Mota SC, Hendriks JA, Ooms ME, Sutmuller RP, et al. Antitumor efficacy of wild-type p53-specific CD4(+) T-helper cells. Cancer Res 2002;62(21):6187-93.

(26) Mumberg D, Wick M, Schreiber H. Unique tumor antigens redefined as mutant tumor-specific antigens. Semin Immunol 1996;8(5):289-93.

(27) Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med 2008;358(25):2698-703.

(28) van der Burg SH, Bijker MS, Welters MJP, Offringa R, Melief CJM. Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. Advanced Drug Delivery Reviews 2006;58(8):916-30.

(29) Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 2008;8(5):351-60.

(30) Zwaveling S, Mota SCF, Nouta J, Johnson M, Lipford GB, Offringa R, et al. Established Human Papillomavirus Type 16-Expressing Tumors Are Effectively Eradicated Following Vaccination with Long Peptides. J Immunol 2002;169(1):350-8.

(31) Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Phase I immunotherapeutic trial with long peptides spanning the e6 and e7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res 2008;14(1):169-77.

(32) Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008;14(1):178-87.

(33) GCIG CA 125 definitions 2005. http://ctep.cancer.gov/resources/gcig/respdef.html

(34) Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92(3):205-16.

(35) Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, et al. Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. Int J Cancer 2008;122(3):486-94.

(36) van der Burg SH, Menon AG, Redeker A, Bonnet MC, Drijfhout JW, Tollenaar RAEM, et al. Induction of p53-specific immune responses in colorectal cancer patients receiving a recombinant ALVAC-p53 candidate vaccine. Clin Cancer Res 2002;8(5):1019-27.

(37) Leffers N, Lambeck AJA, de Graeff P, Bijlsma AY, Daemen T, van der Zee AGJ, et al. Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. Gynecol Oncol 2008;110(3):365-73.

(38) van den Hende M, van Poelgeest MI, van der Hulst JM, de Jong J, Drijfhout JW, Fleuren GJ, et al. Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. Int J Cancer 2008;123(1):146-52.

(39) Svane IM, Pedersen AE, Johnsen HE, Nielsen D, Kamby C, Gaarsdal E, et al. Vaccination with p53-peptide-pulsed dendritic cells, of patients with advanced breast cancer: report from a phase I study. Cancer Immunol Immunother 2004;53(7):633-41.

(40) Speetjens FM, Kuppen PJK, Welters MJP, Essahsah F, Voet van den Brink AMEG, Kallenberg Lantrua MG, et al. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin Cancer Res 2009;15(3):1086-95.

(41) Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. Trends Immunol 2005;26(7):360-6.

(42) Cicinnati VR, Zhang X, Yu Z, Ferencik S, Schmitz KJ, Dworacki G, et al. Increased frequencies of CD8+ T lymphocytes recognizing wild-type p53-derived epitopes in peripheral blood correlate with presence of epitope loss tumor variants in patients with hepatocellular carcinoma. Int J Cancer 2006;119(12):2851-60.

(43) Hoffmann TK, Donnenberg AD, Finkelstein SD, Donnenberg VS, Friebe-Hoffmann U, Myers EN, et al. Frequencies of tetramer+ T cells specific for the wild-type sequence p53(264-272) peptide in the circulation of patients with head and neck cancer. Cancer Res 2002;62(12):3521-9.

(44) Cannistra SA. Cancer of the ovary. N Engl J Med 2004 Dec 9;351(24):2519-29.

(45) Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res 2006;12(3 Pt 1):878-87.

(46) Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med 2008;14(1):28-36.

(47) Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific ecruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942-9.

(48) Wei S, Kryczek I, Edwards RP, Zou L, Szeliga W, Banerjee M, et al. Interleukin-2 administration alters the CD4+FOXP3+ T-cell pool and tumor trafficking in patients with ovarian carcinoma. Cancer Res 2007;67(15):7487-94.

(49) Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56(5):641-8.

(50) Laheru D, Lutz E, Burke J, Biedrzycki B, Solt S, Onners B, et al. Allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor immunotherapy alone or in sequence with cyclophosphamide for metastatic pancreatic cancer: a pilot study of safety, feasibility, and immune activation. Clin Cancer Res 2008;14(5):1455-63.

# Chapter 8

## Longterm clinical and immunological effects of p53-SLP® vaccine in ovarian cancer patients



Leffers N, Hoogeboom BN, Schulze URJ, Wolf R, Hamming LE, van der Zee AGJ, Melief CJ, van der Burg SH, Daemen T, Nijman HW.

*Manuscript in preparation*

## ABSTRACT

### Background

Vaccine-induced p53-specific immune responses were previously reported to be associated with improved response to secondary chemotherapy in small cell lung cancer patients. We investigated longterm clinical and immunological effects of the p53- SLP® vaccine in recurrent ovarian cancer patients.

### Methods

Twenty patients were immunized with the p53-SLP® vaccine between July 2006 and August 2007. Follow-up information on patients was obtained. Clinical responses to secondary chemotherapy after p53-SLP® immunizations as determined by computerized tomography and/or tumour marker levels (CA125) and disease-specific survival were compared with a matched historical control group. Immune responses in post-immunochemotherapy blood samples were analysed by flow cytometry, proliferation assay and/or IFN-γ ELISPOT. Lymphocytes cultered from skin biopsy were analysed by flow cytometry and proliferation assay.

### Results

Seventeen patients were treated with chemotherapy after p53- SLP® immunizations, eight of whom volunteered another blood sample. No differences in clinical response rates to secondary chemotherapy or disease-specific survival were observed between immunized patients and historical controls (p=0.925, resp. p=0.601). P53-specific proliferative responses were observed in 5/8 patients and IFN-γ production in 2/7 patients. Lymphocytes cultured from a prior injection site showing inflammation during chemotherapy did not recognise p53-SLP®.

### Conclusion

Although p53-specific T-cells survive chemotherapy, treatment with the p53- SLP® vaccine does not affect responses to secondary chemotherapy or survival.

## INTRODUCTION

Ovarian cancer, which is generally treated with cytoreductive surgery and platinum-based chemotherapy, is the most frequent cause of death from gynaecological malignancies. In an attempt to improve prognosis by inducing and/or enhancing tumor immune responses, we have recently performed a phase II study with the p53-synthetic long peptide vaccine (p53-SLP®) (1). The vaccine proved safe, well-tolerated and highly immunogenic, but no partial and/or complete clinical responses were observed.

Likewise, many previous p53-based immunotherapeutic strategies have disappointing clinical efficacy although p53-specific immunity was induced (2-7). Interestingly, in patients with small cell lung cancer a trend towards an increased response to secondary chemotherapy was observed after immunisation with dendritic cells virally transduced with the wt-p53 gene (6). Complete or partial responses to second-line chemotherapy were seen in 75% of p53-responders as opposed to 30% of p53-non-responders (p=0.08). Moreover, this clinical response rate of 75% seen after second-line chemotherapy in patients with immunological responses to the p53-transduced dendritic cells (6) is much higher than that seen in historical control groups treated with second-line chemotherapy for progression of disease (6-16%) (8).

Patients with immunological responses to p53-specific immunotherapy may thus be more likely to respond to secondary chemotherapy. Possible explanations for this synergy include 1) up-regulation of p53 in tumour cells in response to chemotherapy thus increasing chances of recognition and destruction by cytotoxic T-cells, and 2) down-regulation of immunosuppressive agents produced by tumour cells, thus enhancing destruction of tumour cells by CTL.

We hypothesised that patients treated with the p53-SLP® vaccine would have a higher response rate to 'secondary' chemotherapy than generally described for palliative chemotherapy for ovarian cancer. Furthermore, we investigated whether p53-specific immunity previously induced by the p53-SLP® vaccine was influenced by 'secondary' chemotherapy.

## PATIENTS AND METHODS

### Elegibility criteria

In a phase II study, epithelial ovarian cancer patients were subcutaneoulsy immunized four times with the p53-SLP® vaccine (1). The vaccine consisted of 10 synthetic long overlapping peptides, spanning amino acids 70-248 of the wt-p53 protein. Clinical response to immunizations was determined 6-9 weeks after the last immunization. Subsequent follow-up information for patients who participated in this phase II study was prospectively collected. Patients treated with chemotherapy after vaccination were invited to give a blood sample to measure the level of p53-specific immune responses. Written informed consent was obtained specifically for the collection of this additional blood sample.

### Evaluation of long term immunogenicity

#### *Lymphocytes and sera*

Blood for immunological assays was obtained at least 4 weeks after chemotherapy. Serum was isolated from clotted blood and cryopreserved. PBMC were isolated from heparinized blood by Ficoll-Paque density centrifugation and freshly used and/or frozen until use in liquid nitrogen.

#### *Antigens (for immunological assays)*

Vaccine peptides were divided into four pools: p1-2 (aa 70-115), p3-4 (aa 102-155), p5-7 (aa 142-203) and p8-10 (aa 190-248). Thirteen overlapping 30-mers spanning the first and last part of wt-p53 protein not included in the vaccine were divided into two pools: aa 1-78 and aa 241-393. Memory recall mix (MRM), a mixture of tetanus toxoid (0.75 limus flocculentius/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands), tuberculine PPD (0.4 µg/mL; Netherlands Vaccin Institute, Bilthoven, the Netherlands) and *C. albicans* (0.015% Greenlabs, Lenoir) was used as a positive control.



#### *IFN-γ ELISPOT assay*

Cryopreserved PBMC obtained before immunization, after immunization and after subsequent chemotherapy were available for 7 patients. P53-specific responses in cryopreserved PBMC obtained before immunization, after four immunizations and after chemotherapy of a single patient were simultaneously determined by IFN-γ ELISPOT as previously described (5;9). PBMC were stimulated with vaccine and non-vaccine p53-peptide pools (10 µg/peptide/mL) or MRM (1:50). A response was considered p53-specific if [(mean number of spots in experimental wells) – (mean number of spots in medium + 2 x SD)] ≥ 10 spots / $10^5$ PBMC. A vaccine-induced response was defined as a p53-specific response which exceeded the pre-existing immune response at least three-fold (10).

***Flow cytometry***

PBMC were evaluated for CD3, CD4, CD8, CD19, CD56 (IQ Products, Groningen, the Netherlands) and FoxP3 expression (eBioscience, San Diego, CA) by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium) according to manufacturer's instructions. Skin biopsy derived lymphocytes were analysed for CD4, CD8, and Foxp3 expression.

***Proliferation assays***

Freshly isolated PBMC were stimulated with vaccine and non-vaccine p53-peptide pools (10 µg/peptide/mL) or MRM (1:156) as previously described (10). Proliferation was considered p53-specific if the stimulation index ≥ 3 and [(mean cpm in experimental well) – (mean cpm in medium + 3x SD medium)] ≥ 0. A vaccine-induced response was defined as a p53-specific response with [(mean cpm in medium + 3x SD medium after treatment) / ((mean cpm in medium + 3x SD medium before immunization)] ≥ 2.

**Evaluation of long-term clinical activity**

***Historical control group***

To evaluate the effect of p53-SLP® treatment on subsequent chemotherapy and survival, a historical control goup was formed with three control patients for each patient treated with the p53-SLP® vaccine. Historical controls were obtained from an anonymous pass-word protected database containing clinicopathological and follow-up data of all epithelial ovarian cancer patients treated with primary debulking surgery according to standard treatment protocols by gynecological oncologists of the University Medical Center Groningen (Groningen, The Netherlands) between May 1985 and May 2006. As no patient identity can be eluded from this computerized database, no further approval from our Institutional Review Board was required for the use of these historical control patients according to Dutch law. P53-SLP® treated patients were matched with historical controls based on FIGO stage, histological tumour type, amount of residual disease after primary debulking surgery, histological grade (in order of importance).

***Evaluation of clinical responses to chemotherapy***

For historical control patients, tumour response to secondline chemotherapy was evaluated based on CA-125 levels and reports of imaging when available. For p53-SLP® treated patients, tumour response to post-immunotherapy chemotherapy was evaluated by serum CA-125 levels (GCIG criteria (11)) and computerized tomography assessed according to RECIST criteria (12) by an experience radiologist (RW).

**Statistical analyses**

Differences in PBMC composition of cryopreserved samples were evaluated with the Wilcoxon signed rank test. Whether matching resulted in similar distributions of clinicopathological characteristics was evaluated by likelihood ratio statistics (categorical variables) or independent samples t-test (normally distributed continous variables). Differences between historical controls and p53-SLP® vaccine treated patients in response rates to chemotherapy for first recurrence were evaluated with Chi-square test. Survival differences were plotted using Kaplan-Meier curves and tested by Log-Rank test. Disease-specific survival was defined as time of diagnosis to date of death of ovarian cancer or last-follow-up.

All analyses were performed using SPSS version 16.0.2 software package for windows (SPSS Inc., Chicago, USA). P values <0.05 were considered significant (tested 2-sided).

**Table 1** Overview of administered p53-SLP® therapy and secondary chemotherapy as clinical responses to therapy.

| Patient | Number of p53-SLP® immunizations | Response to p53-SLP® | Type of post-immunotherapy chemotherapy | Response to post-immunotherapy chemotherapy# | Post-chemotherapy PBMC sample |
|---|---|---|---|---|---|
| P01 | 4 | PD | - | n.a. | n.a. |
| P02 | 4 | PD | C | PR | No |
| P03 | 4 | PD | LP | SD | No |
| P04 | 2 | PD | C, P | PR | No |
| P05 | 4 | PD | C | PR | No |
| P06 | 4 | PD | C, P | PR | Yes |
| P08 | 4 | PD | LP | PD | No |
| P09 | 4 | PD | C, P | PR | Yes |
| P11 | 4 | PD | C, D | PD | Yes |
| P12 | 2 | PD | - | n.a. | n.a. |
| P13 | 4 | PD | LP | PR | No |
| P14 | 4 | PD | - | n.a. | n.a. |
| P15 | 4 | PD | C, P | PR | No |
| P17 | 4 | SD | C, D | CR | Yes |
| P18 | 4 | PD | C | PD | No |
| P19 | 4 | PD | C, D | PR | Yes* |
| P20 | 4 | PD | C, LP | PR | Yes |
| P21 | 4 | PD | C, P | PR | No |
| P22 | 4 | PD | C, D | PR | Yes* |
| P23 | 4 | SD | LP | PD | Yes |

C = carboplatin, D = docetaxel, P = paclitaxel, LP = liposomal doxorubicin, CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, n.a. = not applicable; #based on evaluation of CA125 levels and CT-scans, except for P04/P05 only CA125 and P23 only CT-scan; *second additional sample obtained at time of reactivation of prior injection sites during tertiary chemotherapy for a second recurrence.

## RESULTS

### Patients

Twenty patients were treated with the p53-SLP® vaccine (1). Three patients did not receive subsequent chemotherapy (P01, P12, P14). Responses to p53-SLP® treatment and post-immunotherapy chemotherapy are shown in table 1. Eight patients consented to the donation of an additional blood sample after chemotherapy. No differences in clinicopathological characteristics of immunized patients and matched historical controls were observed (table 2).

### Persistent p53-specific proliferative T-cell responses after chemotherapy

P53-specific proliferative responses were observed in 5/8 patients (63%) after post-immunotherapy chemotherapy as measured by proliferation assay (table 3). In three patients (P17, P20, P23) responses present after the fourth immunization were no longer detactable after subsequent chemotherapy, whereas the opposite held for P09. Persisting responses were frequently higher after chemotherapy than after the fourth immunization. Interestingly, after chemotherapy proliferative responses to aa 241-393, part of the p53-protein not covered by the p53-SLP® vaccine, increased in number as well as strength.

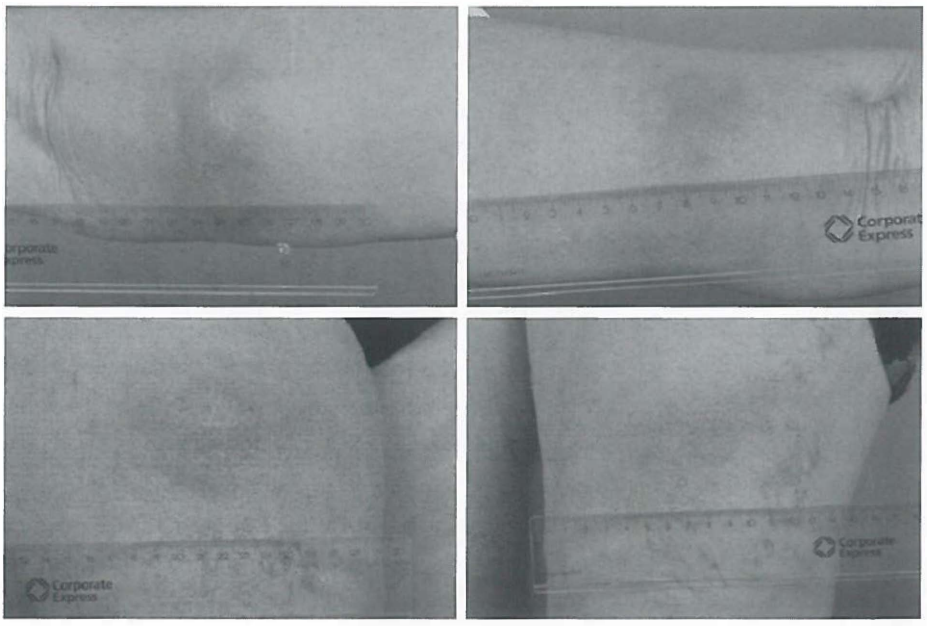

**Fig. 1** Spontaneous loco-regional inflammatory responses during chemotherapy for secondary recurrence at all four injection sites.

An IFN-γ ELISPOT was performed for seven patients to evaluate whether these p53-specific cells produced IFN-γ (table 4). Comparable to the previously published results (1), no significant differences existed in composition of cells obtained before immunization, after immunization and after chemotherapy as analysed simultaneously by flow cytometry (data not shown). After chemotherapy, two patients (29%) showed p53-specific IFN-γ production in response to stimulation of PBMC with vaccine peptides p3-p4 and p8-p10 (P06, P20), whereas no such responses were observed in these patients after four immunizations. In patients with responses against vaccine peptides after immunotherapy (P11, P17, P22) no IFN-γ producing T-cells could be detected after subsequent chemotherapy.

**Table 2** Clinicopathological characteristics of p53-SLP treated patients and matched historical controls

| | P53-SLP® (n=20) | | Historical controls (n=60) | | p-value |
|---|---|---|---|---|---|
| *Age at diagnosis* | | | | | |
| Mean (SD) | 52.4 | (8.8) | 58.7 | (11.8) | 0.032 |
| *FIGO stage* | | | | | |
| IC | 1 | (5.0%) | 3 | (5.0%) | 0.219 |
| IIc | 1 | (5.0%) | 3 | (5.0%) | |
| IIIB | 2 | (10.0%) | 0 | (0.0%) | |
| IIIC | 14 | (70.0%) | 48 | (80.0%) | |
| IV | 2 | (10.0%) | 6 | (10.0%) | |
| *Histology* | | | | | |
| Serous | 13 | (65.0%) | 39 | (65.0%) | 1.000 |
| Mucinous | 1 | (5.0%) | 3 | (5.0%) | |
| Endometrioid | 3 | (15.0%) | 9 | (15.0%) | |
| Clear cell | 3 | (15.0%) | 9 | (15.0%) | |
| *Differentiation* | | | | | |
| Grade I | 2 | (10.0%) | 6 | (10.0%) | 0.263 |
| Grade II | 5 | (25.0%) | 20 | (33.3%) | |
| Grade III | 13 | (65.0%) | 29 | (48.3%) | |
| unknown | 0 | (0.0%) | 5 | (8.3%) | |
| *Residual disease* | | | | | |
| <2 cm | 10 | (50.0%) | 29 | (48.3%) | 0.808 |
| ≥2 cm | 6 | (30.0%) | 22 | (36.7%) | |
| unknown | 4 | (20.0%) | 9 | (15.0%) | |

FIGO = international federation of Gynecology and Obstetrics

**Reactivation of injection sites is not caused by p53-specific T-lymphocytes**

Reactivation of prior injection sites during chemotherapy was reported by several patients (figure 1). For two patients (P19, P22) an additional blood sample was obtained for IFN-γ ELISPOT at the time of reactivation during chemotherapy for a second recurrence, in addition to the sample obtained after the first course of post-immunotherapy chemotherapy. Similar to the results after the first course of post-immunotherapy, no p53-specific responses were observed for P19 at the time of reactivation of the vaccine injection sites. Although P22 had p53-specific responses against p3-p4, p5-p7 and p8-p10, only responses against p8-p10 could be considered vaccine-induced (43, 57 and 189 specific spots / $10^5$ cells respectively).

P22 also consented to a skin biopsy taken at a reactivated immunization site. This biopsy was used for the culture of T-lymphocytes as previously described (1), yielding 12.3 x $10^6$ lymphoyctes after 4 weeks of culturing ($CD4^+$ 15%, $CD8^+$ 5%). Cells were subsequently used for a 6-day proliferation assay. The lymphocytes cultured from the biopsy taken from the reactivated injection site were not p53-specific, indicating that reactivation of prior injection sites is not a p53-specific event (data not shown).

**Longterm clinical activity**

Information on clinical response to secondline chemotherapy was available for 30 historical controls. Response rates to secondline chemotherapy were similar for p53-SLP® vaccine treated patients and historical controls (CR/PR 60.0% vs. 61.5%, p=0.925). Likewise, median disease-specific survival did not differ between p53-SLP® treated ovarian cancer patients and historical controls (median DSS 44.0 vs. 47.4 months, p=0.601). Inclusion of only those patients who received all four p53-SLP SLP® immunizations or exclusion of patients not treated with chemotherapy after immunotherapy did not result in differences in DSS either.

**Table 3** Vaccine-Induced P53-specific Immune Responses in PBMC of Ovarian Cancer Patients Immunized with the P53-SLP Vaccine as Analyzed by proliferation assay after four immunizations and after subsequent chemotherapy

| | After four immunizations | | | | | | After chemotherapy | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Vaccine peptides | | | | Non-vaccine peptides | | Vaccine peptides | | | | Non-vaccine peptides | |
| Patient[1] | p1-p2[2] | p3-p4 | p5-p7 | p8-p10 | aa 1-78 | aa 241-393 | p1-p2 | p3-p4 | p5-p7 | p8-p10 | aa 1-78 | aa 241-393 |
| P06 | - | 4.6[3] | - | 3.6 | - | - | 5.4 | 5.2 | 176.6 | - | - | 8.7 |
| P09 | - | - | - | - | - | - | - | 43.8 | 147.9 | - | - | 92.0 |
| P11 | na | na | na | na | na | na | - | 9.0 | 16.1 | - | - | 11.1 |
| P17 | - | - | - | 10.3 | - | - | - | - | - | - | - | - |
| P19 | - | 10.6 | 12.9 | - | - | - | - | 43.2 | 42.9 | 109.9 | - | 82.9 |
| P20 | 6.4 | 28.3 | - | 11.7 | - | 5.9 | - | - | - | - | - | - |
| P22 | - | - | 23.9 | 16.7 | - | 46.8 | - | - | 9.4 | 33.4 | - | 60.8 |
| P23 | 101.3 | 2.5 | - | - | 2.4 | - | - | - | - | - | - | - |

[1]Patients analysed for p53-specific responses after four immunizations and after subsequent chemotherapy by proliferation assay. [2]The pool of p53 vaccine peptides used to stimulate patient-derived PBMC *in vitro* for 6 days. [3]Only vaccine-induced p53-specific responses are shown (see definition in Material and Methods). [3]Responses are depicted as the mean of corrected p53-induced proliferation after four immunizations or chemotherapy divided by the mean of corrected p53-induced proliferation before immunization. - = no vaccine-induced p53-specific response; na = PBMC were not available.

**Table 4** Vaccine-Induced P53-specific Immune Responses in PBMC of Ovarian Cancer Patients Immunized with the P53-SLP Vaccine as Analyzed by IFN-γ ELISPOT

| | Before immunization | | | | | | After four immunizations | | | | | | After chemotherapy | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Vaccine peptides | | | | Non-vaccine peptides | | Vaccine peptides | | | | Non-vaccine peptides | | Vaccine peptides | | | | Non-vaccine peptides | |
| Patient[1] | p1 p2[2] | p3 p4 | p5 p7 | p8 p10 | Aa 1-78 | Aa 241-393 | p1 p2 | p3 p4 | p5 p7 | p8 p10 | Aa 1-78 | Aa 241-393 | p1 p2 | p3 p4 | p5 p7 | p8 p10 | Aa 1-78 | Aa 241-393 |
| P06 | - | - | - | - | - | - | - | - | - | - | - | - | - | 24 | - | 27 | - | 24 |
| P09 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| P11 | - | - | - | - | - | - | - | - | 16 | 41 | - | - | - | - | - | - | - | - |
| P17 | - | - | - | - | - | - | - | - | - | 21 | - | - | - | - | - | - | - | - |
| P19 | - | - | - | - | nd | nd | - | - | - | - | - | 39 | - | - | - | - | - | 30 |
| P20 | - | - | - | - | - | - | - | - | - | - | - | 11 | - | 32 | - | 14 | - | 15 |
| P22 | - | 18 | 42 | 59 | - | - | - | 41 | 48 | 38 | 11 | 37 | - | - | - | - | - | - |
| P23 | - | - | - | - | - | 12 | - | - | - | - | - | - | - | - | - | - | - | - |

[1]Patients analysed for p53-specific responses before, after four immunizations and after subsequent chemotherapy by IFN-γ ELISPOT. [2]The pool of p53 vaccine peptides used to stimulate patient-derived PBMC *in vitro* for 4 days. [3]Only p53-specific responses are shown (see definition in Material and Methods). Responses are depicted as number of specific spots per $10^5$ PBMC (mean of experimental wells – (mean + 2xSD) of medium control). - = no p53-specific response; nd = no or insufficient PBMC were available.

## DISCUSSION

The p53-SLP® vaccine was recently shown to induce p53-specific T-cell responses in ovarian cancer patients. We investigated longterm immunological and clinical effects of the p53-SLP® vaccine as it has been suggested that responses to chemotherapy might improve in patients with vaccine-induced immune responses (6). Despite the presence of p53-specific immune responses in patients treated with the p53-SLP® vaccine, neither clinical responses to chemotherapy for recurrent disease nor survival differed from the response rates and survival of historical controls. After chemotherapy for recurrent disease subsequent to p53-SLP® immunotherapy, p53-specific immune responses could be detected in 6/8 patients willing and able to give an additional blood sample. Although reactivation of inflammatory reactions at prior injection sites during chemotherapy for a second recurrence was accompanied by circulating p53-specific T-cells recognizing p8-p10 in one patient, no locally active p53-specific T-cells could be detected.

A trial of p53 immunization in small cell lung cancer patients showed a trend towards improved response rates to secondary chemotherapy for immunological responders (6). As there was no distinct group of non-responders in our previous trial (1), a similar comparison between immunologial responders and non-responders could not be made. We therefore compared clinical response rates to chemotherapy for recurrent disease with a historical control group. No differences in response rates to chemotherapy were observed, which may be attributed to several causes. Firstly, although an attempt was made to match p53- SLP® treated patient with historical controls based on some well-known prognostic factors, such retrospective comparisons remain prone to selection bias. To obtain a truly reliable insight in the effect of p53-SLP® treatment on chemotherapy and survival, a randomized controlled trial should be performed with patients allocated to p53-SLP treatment or a control arm receiving no or placebo treatment. Secondly, one could argue that the likelihood of improved clinical responses after p53-SLP® treatment is reduced as immunization with the p53-SLP® vaccine was shown to induce predominantly Th2 $CD4^+$ T-cells, which are unlikely to contribute to effective anti-tumour responses (1). Moreover, after chemotherapy, the number of patients with IFN-$\gamma$ producing p53-specific T-cells was substantially lower than the number of patients with p53-specific proliferating T-cells. This suggests that especially IFN-$\gamma$ producing Th1 $CD4^+$ T-cells are prone to destruction by chemotherapy, whereas Th2 $CD4^+$ T-cells seem to be less affected. Such an effect of chemotherapy on Th1 and Th2 T-cells was previously described for breast cancer patients (13). Whether this also holds to be true in the current study remains to be confirmed by evaluation of cytokine profiles of p53-specific T-cells after chemotherapy.

Interestingly, several patients reported reactivation of immunization sites during chemotherapy for recurrent disease. P53-specific immune responses in blood samples obtained at the time of progression were observed in one of two patients. In a skin biopsy of the injection site from this patient, no p53-specific T-cells could be detected. This suggests that the inflammatory reactions at the immunization sites during chemotherapy may be an immune responses enhanced by Montanide ISA51, the adjuvant used in the p53-SLP® vaccine. Next to its depot function, this water-in-oil emulsion allows slow release of antigens, and has been reported to promote inflammation (innate immune responses) and recruitment of antigen presenting cells as well as lymphocytes (adaptive immune responses) (14).

In summary our results indicate that immunotherapy of recurrent ovarian cancer with the p53-SLP® vaccine does not affect responses to subsequent chemotherapy or prognosis. Furthermore, we show that vaccine-induced p53-specific T-cells can still be detected after chemotherapy.



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## REFERENCES


(1) Leffers N, Lambeck AJ, Gooden MJ, et al. Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial. Int J Cancer 2009.

(2) Herrin V, ehrens RJ, chtar M, onahan S, ernstein S, rent-Steele T et al. Wild-type p53 peptide vaccine can generate a specific immune response in low burden ovarian adenocarcinoma. American Society of Clinical Oncology, annual meeting 2003: abstract 660.

(3) Herrin VE, Achtar MS, Steinberg SM, Whiteside TL, Whiteside E, Weickowski E et al. A randomized phase II p53 vaccine trial comparing subcutaneous direct administration with intravenous peptide-pulsed dendritic cells in high risk ovarian cancer patients. American Society of Clinical Oncology, annual meeting 2007: abstract 3011.

(4) Svane IM, Pedersen AE, Johansen JS, et al. Vaccination with p53 peptide-pulsed dendritic cells is associated with disease stabilization in patients with p53 expressing advanced breast cancer; monitoring of serum YKL-40 and IL-6 as response biomarkers. Cancer Immunol Immunother 2007; 56:1485-1499.

(5) van der Burg SH, Menon AG, Redeker A, et al. Induction of p53-specific immune responses in colorectal cancer patients receiving a recombinant ALVAC-p53 candidate vaccine. Clin Cancer Res 2002; 8:1019-1027.

(6) Antonia SJ, Mirza N, Fricke I, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res 2006; 12:878-887.

(7) Svane IM, Pedersen AE, Johnsen HE, et al. Vaccination with p53-peptide-pulsed dendritic cells, of patients with advanced breast cancer: report from a phase I study. Cancer Immunol Immunother 2004; 53:633-641.

(8) Davies AM, Evans WK, Mackay JA, Shepherd FA. Treatment of recurrent small cell lung cancer. Hematol Oncol Clin North Am 2004; 18:387-416.

(9) Lambeck A, Leffers N, Hoogeboom BN, et al. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007; 121:606-614.

(10) Welters MJ, Kenter GG, Piersma SJ, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008; 14:178-187.

(11) GCIG CA 125 definitions 2005. http://ctep.cancer.gov/resources/gcig/respdef.html.

(12) Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92:205-216.

(13) Ferrari S, Malugani F, Rovati B, Porta C, Riccardi A, Danova M. Flow cytometric analysis of circulating dendritic cell subsets and intracellular cytokine production in advanced breast cancer patients. Oncol Rep 2005; 14:113-120.

(14) Kaeberle ML. Function of carriers and adjuvants in induction of immune responses. In: Nervig RM, Gough PM, Kaeberle ML, Whetstone CA, editors. Adv. in carriers and adjuvants for veterinary biologics. Iowa State University press, 1986: 11-23.

# Chapter 9

## Summarizing discussion and future perspectives



Partly based on:
Multimodality treatment warranted for ovarian cancer: immunotherapy, a prerequisite to improve prognosis for this vicious disease.

Leffers N, Daemen T, van der Zee AGJ, Nijman HW.

Immunotherapy 2009;1(2):163-5.

## Introduction

Ovarian cancer is generally treated with cytoreductive surgery followed by chemotherapy. As prognosis depends heavily on the amount of residual disease after primary debulking surgery, involvement of experienced gynaecological oncologists in the treatment of ovarian cancer patients improves survival (1). Furthermore, with the introduction of taxanes in the mid-nineties, survival of (in)completely debulked advanced stage patients has improved (2). Despite these developments in treatment, ovarian cancer remains the most lethal gynaecological malignancy. To improve prognosis, new therapeutic modalities are currently being explored. This thesis focuses on immunotherapy as a therapeutic option for ovarian cancer. Results of a clinical immunotherapy trial and translational research supporting this modality as well as highlighting potential pitfalls are presented.

## Immunotherapy for ovarian cancer

The development of immunotherapy for cancer logically ensues from the observation of tumour-specific immunity and prognostic influence of tumour-infiltrating lymphocytes. In **chapter two**, the prognostic influence of three types of lymphocytes infiltrating primary and metastatic tumour tissue was evaluated in 306 ovarian cancer patients. $CD8^+$ cytotoxic T-lymphocytes were observed in nearly all specimens, whereas $FoxP3^+$ regulatory and $CD45R0^+$ memory/effector T-lymphocytes both were present in approximately 50% of primary tumour samples. The presence of high numbers of $CD8^+$ T-lymphocytes was an independent predictor of improved survival. Although regulatory T-lymphocytes are considered to negatively influence survival in cancer patients, no direct impact on survival was observed for ovarian cancer patients. It was however noted that high numbers of cytotoxic T-cells relative to regulatory T-cells was also an independent predictor of improved survival. This favourable influence of tumour-infiltrating lymphocytes, especially cytotoxic T-lymphocytes, on ovarian cancer prognosis combined with prior observations of tumour-specific immune responses in ovarian cancer patients (3-8) support the exploration of immunotherapy for this disease.

Thus far, many different strategies targeting many different tumour-antigens have been employed in an attempt to induce clinically effective antigen-specific anti-tumour immune responses in ovarian cancer patients (summarized in **chapter 6**). Most tumour-antigen specific vaccines induce immunological responses, although differences in immunological response rates and potency are substantial. A new strategy in immunotherapy is the immunization with synthetic long peptides (SLP®). Advantages of immunization with SLP® are the reliance on cross-presentation by dendritic cells, the potential presence of epitopes for both cytotoxic and helper T-cells within a single SLP® and the increased duration of epitope presentation (9;10). Compared to strategies using short peptides or whole protein, overlapping long peptides are more effective in priming sustained immune responses (11;12). Based on these promising characteristics, we performed an immunization study

using SLP encoding for part of the p53-protein, described in **chapter 7**. The p53-SLP® vaccine, containing 10 overlapping SLP® and the adjuvant Montanide ISA51, was well-tolerated and induced p53-specific T-cells in all patients immunized four times as measured in peripheral blood. Encouragingly, p53-specific T-cells were also observed in skin biopsies obtained from the fourth immunization site, indicating that induced cells were capable of migration. Comparable to most immunotherapy studies in ovarian cancer, clinical responses were not observed. This is not surprising given that induced cells were helper T-lymphocytes predominantly producing Th2 cytokines. This observation emphasizes a potential drawback of SLP®-vaccines, i.e. dependence on co-stimulatory adjuvants. To promote Th1 polarization, Toll-like receptor (TLR) agonists could be applied as adjuvants as these ligands activate DC maturation, induce secretion of inflammatory cytokines by innate immune cells and have been suggested to help overcome tolerance to self-antigens as well as to promote responses to tumour antigens (13).

Although vaccine-induced p53-specific immune responses seem to be associated with improved clinical responses to secondary chemotherapy in small cell lung cancer patients, in **chapter 8** we show that treatment with the p53-SLP® vaccine does not improve clinical responses to subsequent chemotherapy or prognosis of ovarian cancer patients. Furthermore, although proliferating p53-specific immune responses can be detected after chemotherapy in the majority of patients, especially Th1 $CD4^+$ T-lymphocytes seem to be negatively influenced by chemotherapy. Whether the addition of potent Th1 polarizing adjuvants to the p53-SLP® vaccine sufficiently improves induction of Th1 T-cells to overcome this negative effect of chemotherapy needs to be further explored.

## Challenges facing immunotherapy

There are several types of obstacles that need to be overcome to reach clinical effectiveness of immunotherapy in ovarian cancer, e.g. patient specific, tumour-cell specific and research specific issues.

Firstly, patients participating in immunotherapeutic trials frequently have gross recurrent disease, which may simply be too substantial to be eradicated by an activated immune system. Better results might be obtained by immunizing patients with pre-malignant disease(a stage that currently cannot be detected in ovarian cancer) or concurrent with or shortly after successful primary therapy.

Secondly, the success of immunotherapy for (ovarian) cancer may also be hampered by strategies 'employed' by tumour cells to escape destruction by the immune system. These approaches have been categorized as: evasion of recognition by the immune system (e.g., down-regulation of MHC class I, tumour antigens and/or other components of the antigen presentation machinery); expression and/or secretion of immunosuppressive substances (e.g., IDO, IL-10 and TGF-β); up-regulation of negative co-stimulatory signals (e.g., PD-L1 and Fas-L); recruitment of immunosuppressive cells (e.g., regulatory T cells and myeloid-

derived suppressor cells); and interference with extravasation and/or homing of lymphocytes (14-17). Evasion of recognition by the immune system was described in chapters 3 & 4. In **chapter 3** expression of a tumour-antigen was investigated simultaneously with down-regulation of MHC-class I in 329 ovarian cancer patients. When tumours of patients expressing the tumour-antigen p53 down-regulated MHC class I expression, median survival of patients was diminished by 10.8 months compared to patients with normal MHC class I expression. For patients not expressing the p53-antigen, no such survival advantage of normal MHC class I expression was observed. In **chapter 4** the MHC class I dependent antigen processing and presentation pathway was more closely examined. Using tissue microarrays containing tumour tissue of 232 ovarian cancer patients, nine components of this pathway were examined by immunohistochemistry. In addition to the diminished survival observed in patients with down-regulation of MHC class I expression, disease-specific survival was also negatively influenced by expression of the constitutive proteasome component MB1. Therapy with proteasome inhibitors may thus be advantageous for patients with tumours containing the constitutive proteasome. Conversely, proteasome inhibition may be harmful in patients with a preponderance of the immunoproteasome. Careful selection of patients eligible for proteasome treatment and/or development of more selective proteasome inhibitors might therefore be advisable. In chapter 4 it was also observed that down-regulation of MHC class I molecules was strongly associated with down-regulation of nearly all other components examined, suggesting a seemingly coordinated down-regulation of the MHC class I dependent antigen processing and presentation pathway. In **chapter 5** in which differential gene expression and pathway activation between tumours containing few and many intra-epithelial lymphocytes were compared, the antigen processing and presentation pathway was once more identified. A selection of genes in this pathway was shown to be enriched in tumours with high numbers of infiltrating lymphocytes. This was subsequently validated at the protein level by means of immunohistochemistry. Targeted therapies aiming at enhancing expression of the pathways associated with tumour-infiltrating lymphocytes, e.g. the antigen processing and presentation pathway, may be a lucrative strategy to increase likelihood of clinical responses to immunotherapy. In light of these and numerous other immune evasion strategies, it can be inferred that the key to success, probably lies in combinatorial strategies (i.e., immunotherapy to induce anti-tumour immune responses together with drugs that target the above-mentioned immune-escape mechanisms) (16).



Lastly, a more practical challenge facing the scientific community involved in immunotherapy calls for attention. In **chapter 6** an overview was given of antigen-specific active immunotherapy trials performed in ovarian cancer patients over the last 17 years. A major limitation of these trials uncovered by this review was the lack of uniformity in trial conduct, clinical and immunological response definitions and trial reporting. To allow for conclusive comparison of immunological and clinical effectiveness, it is deemed imperative that those who conduct immunotherapy trials adopt universally accepted immunological and clinical response definitions, guidelines for adverse events reporting, as well as internationally

accepted directives for trial conduct and reporting. Additionally, in studies with clinical responses an attempt should be made to identify what immunological results best predict these clinical responses. For example, in vaccines developed against infections primarily cleared by T-lymphocytes (e.g. HIV, malaria, tuberculosis), a strong correlation between clinical effectiveness and multi-functionality of Th1 and $CD8^+$ T-cells has been observed. Protection improved in the presence of IFN-$\gamma^+$TNF$^+$IL-2$^+$ T-lymphocytes, compared to other combinations of IFN-$\gamma$, TNF and IL-2 production (18;19). Whether such observations also hold for lymphocytes induced by cancer vaccines, needs to be examined. Ultimately, the immunological assay with the best predictive value for clinical responses to cancer immunotherapy should consistently be included in immunomonitoring of trials.

## Future Perspectives

The immunotherapy trial described in chapter 7 of this thesis specifically targets the tumour antigen p53. As malignant cells may down-regulate tumour antigens in an attempt to escape destruction by the immune system, immunizing patients with more than one tumour antigen in an individualized approach could be a valuable method. Alternatively, a cocktail directed towards multiple well-defined tumour-antigens could be contrived, containing antigenic targets present in the majority of patients. A possible drawback of both methods mentioned may be immunodominance of specific antigens resulting in antigenic competition between antigens and interference with effectiveness. Conversely, a putative advantage of such a multi-antigen cocktail also containing p53-SLP®, could be the induction of p53-specific "universal' helper T-cells that facilitate stimulation of cytotoxic T-cells directed towards other tumour antigens targeted by the cocktail vaccine, as was previously observed for NY-ESO-1-specific helper T-cells (20).

Although SLP®-vaccines are highly capable of inducing immune responses, similar to other homologous prime-boost immunization strategies, responses seem to decrease after repeated immunizations (21). Should this decrease in responses measured in the circulation be attributed to hypo-responsiveness rather than to migration of effector cells to antigen-expressing tissues, modification of the immunization regimen to a heterologous prime-boost schedule could be rewarding. For instance, sequential *in vivo* immunization with a plasmid and short peptides encoding for a single tumour-antigen, resulted in the induction of higher numbers of T-cells, production of larger amounts of chemokines and pro-inflammatory cytokines, and better recognition of tumour cells than repeated immunization with either component alone (22). Priming with a viral vector encoding a target antigen of choice, e.g. recombinant Semliki Forest Virus (23), followed by immunization with SLP® encoding the same antigen, might thus be an interesting approach to boost tumour-antigen specific immune responses.

Next to modifications in immunization strategy, immunotherapy could also be combined with or given adjuvantly to classic anti-cancer therapy. Chemotherapeutic agents currently or

previously used in the primary treatment of ovarian cancer (i.e., docetaxel or cyclophosphamide) have been shown to enhance antigen-specific immune responses to immunotherapy (24;25). Possible explanations include suppression of regulatory T-cells and increased cross-presentation due to antigen release from tumour debris. Furthermore, although radiotherapy is not part of standard treatment for ovarian cancer, localised radiotherapy of tumours at non-tumoritoxic dosages resulted in MHC class I upregulation by tumour cells as well as in control of distant non-irradiated metastases in *in vivo* models (personal communication Prof. J. Neefjes, Netherlands Cancer Institute). Ionizing radiation therapy not only facilitates antigen presentation, it also induces secretion of chemokines involved in the recruitment of effector T-cells, such as CXCL16 (26). These observations strongly support multimodality treatment incorporating classic anti-cancer treatment modalities.

In addition to classic anti-cancer therapy with immunomodulatory capacities and heterologous prime-boost immunizations, targeted therapies addressing immune escape mechanisms may be a valuable contribution to multimodality treatment of ovarian cancer. Promising results have, for example, been attained by the addition of an endothelial receptor B antagonist to previously clinically ineffective immunotherapeutic strategies in murine models, thereby promoting lymphocyte recruitment and tumour responses (14). Other agents that may possibly be included in such a multimodality approach include agents interfering with negative co-stimulatory signals (e.g., anti-CTLA-4 antibodies) that induced tumour-specific immune cells independent of antigen-specific immunotherapy in metastatic melanoma patients (27), and therapies resulting in MHC class I upregulation (e.g. 5-aza-2´-deoxycytidine (28)). In addition, drugs that directly target processes pivotal to tumour growth and survival may also contribute to such a regimen, for example, agents that disrupt tumour vasculature or inhibit angiogenesis (e.g., bevacuzimab) (29).

In summary, although significant advances have been made in the field of immunotherapy for ovarian cancer, important challenges remaining are (i) when to immunize, (ii) what antigens are best targeted, (iii) what vaccine-type combined with what adjuvant has the best trade-off between immunogenicity and clinical applicability and (iv) how to overcome immune escape by tumour cells. For the future, we envision a multimodality approach for ovarian cancer where immunotherapy directed towards multiple well-defined antigens is combined with cytoreductive surgery, (immunomodulatory) classic chemotherapy and targeted drugs intended to prevent and/or reverse immune escape by the tumour.

## REFERENCES


(1) Engelen MJ, Kos HE, Willemse PH, et al. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. Cancer 2006;106:589-598.

(2) McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996;334:1-6.

(3) Chiriva-Internati M, Wang Z, Salati E, Timmins P, Lim SH. Tumor vaccine for ovarian carcinoma targeting sperm protein 17. Cancer 2002;94:2447-2453.

(4) Lambeck A, Leffers N, Hoogeboom BN, et al. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007;121:606-614.

(5) Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 1995;181:2109-2117.

(6) Ioannides CG, Fisk B, Jerome KR, Irimura T, Wharton JT, Finn OJ. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J Immunol 1993;151:3693-3703.

(7) Qian F, Gnjatic S, Jager E, et al. Th1/Th2 CD4+ T cell responses against NY-ESO-1 in HLA-DPB1*0401/0402 patients with epithelial ovarian cancer. Cancer Immun 2004;4:12.

(8) Yamada A, Kawano K, Harashima N, et al. Study of HLA class I restriction and the directed antigens of cytotoxic T lymphocytes at the tumor sites of ovarian cancer. Cancer Immunol Immunother 1999;48:147-152.

(9) Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 2008;8:351-360.

(10) Obst R, van Santen HM, Melamed R, Kamphorst AO, Benoist C, Mathis D. Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation. Proc Natl Acad Sci U S A 2007;104:15460-15465.

(11) Bijker MS, van den Eeden SJF, Franken KL, Melief CJM, Offringa R, van der Burg SH. CD8+ CTL Priming by Exact Peptide Epitopes in Incomplete Freund's Adjuvant Induces a Vanishing CTL Response, whereas Long Peptides Induce Sustained CTL Reactivity. J Immunol 2007;179:5033-5040.

(12) Zhang H, Hong H, Li D, et al. Comparing pooled peptides with intact protein for accessing cross-presentation pathways for protective CD8+ and CD4+ T cells. J Biol Chem 2009;284:9184-9191.

(13) Conroy H, Marshall NA, Mills KH. TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. Oncogene 2008;27:168-180.

(14) Buckanovich RJ, Facciabene A, Kim S, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med 2008;14:28-36.

(15) Croci DO, Zacarias Fluck MF, Rico MJ, Matar P, Rabinovich GA, Scharovsky OG. Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. Cancer Immunol Immunother 2007;56:1687-1700.

(16) Begley J, Ribas A. Targeted therapies to improve tumor immunotherapy. Clin Cancer Res 2008;14:4385-4391.

(17) Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;27:5904-5912.

(18) Darrah PA, Patel DT, De Luca PM, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 2007;13:843-850.

(19) Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008;8:247-258.

(20) Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med 2008;358:2698-2703.

(21) Svane IM, Pedersen AE, Johnsen HE, et al. Vaccination with p53-peptide-pulsed dendritic cells, of patients with advanced breast cancer: report from a phase I study. Cancer Immunol Immunother 2004;53:633-641.

(22) Smith KA, Tam VL, Wong RM, et al. Enhancing DNA vaccination by sequential injection of lymph nodes with plasmid vectors and peptides. Vaccine 2009;27:2603-2615.

(23) de Mare A, Lambeck AJ, Regts J, et al. Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. Gene Ther 2008;15:393-403.

(24) Garnett CT, Schlom J, Hodge JW. Combination of docetaxel and recombinant vaccine enhances T-cell responses and antitumor activity: effects of docetaxel on immune enhancement. Clin Cancer Res 2008;14:3536-3544.

(25) Laheru D, Lutz E, Burke J, et al. Allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor immunotherapy alone or in sequence with cyclophosphamide for metastatic pancreatic cancer: a pilot study of safety, feasibility, and immune activation. Clin Cancer Res 2008;14:1455-1463.

(26) Matsumura S, Wang B, Kawashima N, et al. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. J Immunol 2008;181:3099-3107.

(27) Yuan J, Gnjatic S, Li H, et al. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. Proceedings of the National Academy of Sciences 2008;105:20410-20415.

(28) Adair SJ, Hogan KT. Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. Cancer Immunol Immunother 2008.

(29) Spannuth WA, Sood AK, Coleman RL. Angiogenesis as a strategic target for ovarian cancer therapy. Nat Clin Pract Oncol 2008;5:194-204.

# Chapter 10

## Nederlandse samenvatting voor de leek

## INLEIDING

### Eierstokkanker

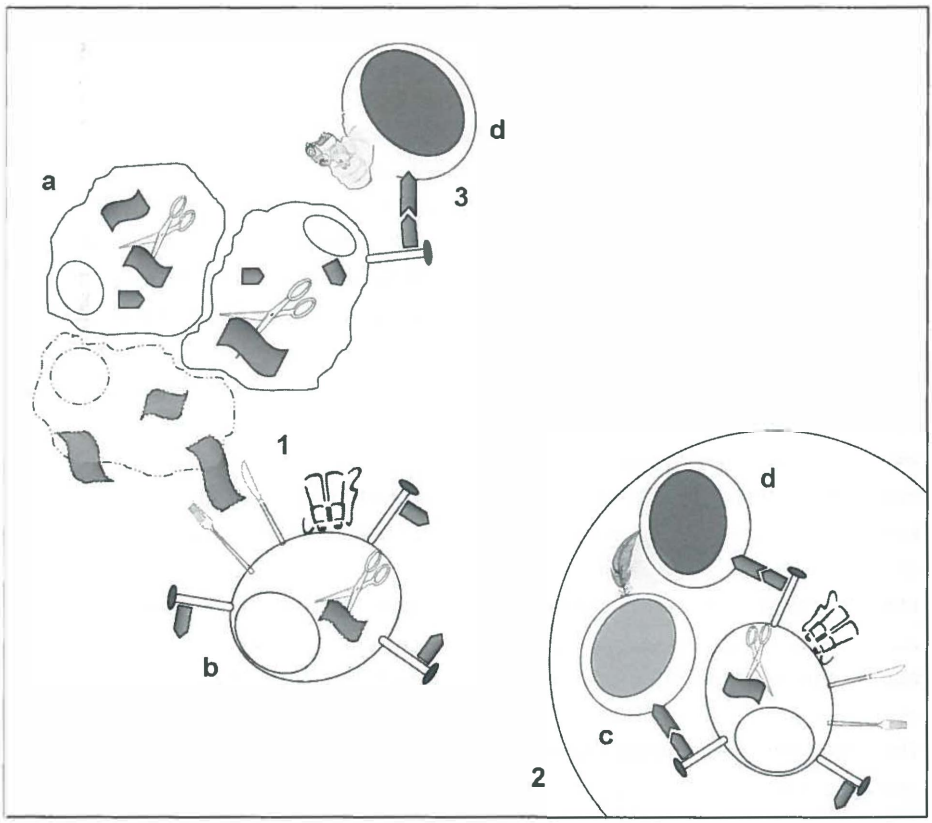
Eierstokkanker is de verzamelnaam voor kwaadaardige gezwellen aan de eierstokken. Het overgrote deel van deze kwaadaardige gezwellen ontstaat uit een enkele cellaag die de eierstok bekleedt, het epitheel. Behalve uit het epitheel kan eierstokkanker ook ontstaan uit bind- en steunweefsel en uit eicellen aanwezig in de eierstok. Voor dit proefschrift geldt dat waar wordt gesproken van eierstokkanker, eierstokkanker van epitheliale oorsprong wordt bedoeld.

Eierstokkanker behoort tot de tien meest voorkomende vormen van kanker bij vrouwen in Nederland. De diagnose werd in 2006 bij 1092 Nederlandse vrouwen gesteld. Aanvankelijk gaat eierstokkanker gepaard met weinig klachten. Klachten treden doorgaans pas op wanneer de kanker zich uit heeft gebreid tot aangrenzende organen en structuren (zoals darmen, blaas, buikvlies). De behandeling van eierstokkanker bestaat gewoonlijk uit een operatie waarbij zoveel mogelijk kankerweefsel wordt verwijderd. Aansluitend worden patiënten met uitzaaiingen in de buikholte nabehandeld met chemotherapie. Hoewel de kanker over het algemeen goed lijkt te reageren op deze behandeling, komt eierstokkanker bij de meeste patiënten met uitzaaiingen in de buikholte ten tijde van de diagnose toch terug. Er wordt dan opnieuw behandeld met chemotherapie. Omdat er (nog) geen behandeling bestaat waarmee teruggekomen eierstokkanker genezen kan worden, is het doel van hernieuwde behandeling het bereiken van een zo lang mogelijke ziektevrije periode. Ondanks de huidige behandeling, blijft eierstokkanker binnen de gynaecologische kankers de meest voorkomende doodsoorzaak: na vijf jaar zijn 80-90% van de vrouwen met uitgezaaide ziekte ten tijde van de initiële diagnose overleden. Voor vrouwen zonder uitzaaiingen in de buikholte, geldt een betere levensverwaching: na 5 jaar is nog 60-100% in leven.

### De rol van het afweersysteem in kanker

Het afweersysteem heeft als belangrijkste taak het herkennen en aanvallen van indringers zoals virussen en bacteriën. Dat het afweersysteem ook een rol speelt bij kanker blijkt uit het feit dat de aanwezigheid van afweercellen in kanker sterk samenhangt met een goede levensverwachting. Het afweersysteem bestaat grofweg uit twee intensief samenwerkende onderdelen, de aangeboren afweer en de aangeleerde afweer (figuur 1). Belangrijke spelers van de aangeleerde afweer zijn de zogenaamde T-cellen, de soldaten van het afweersysteem. De T-cellen herkennen vlaggetjes, de zogenaamde antigenen, gehesen in vlaggenmasten op het oppervlak van (kanker)cellen, beter bekend als HLA of MHC moleculen. Voordat ze tot actie kunnen overgaan, moeten ze echter worden geactiveerd door cellen van de aangeboren afweer, de zogenaamde antigeenpresenterende cellen. Antigeenpresenterende cellen zijn de verkenners van het immuunsysteem. Ze

herkennen afvalstoffen van (stervende) kankercellen, eten deze op en hijsen stukjes hiervan in de vlaggenmast op hun celoppervlak. Vervolgens verplaatsen deze verkenners zich naar de lymfeklieren, de kazernes van het afweersysteem, alwaar ze de gestationeerde T-cellen over hun missie informeren. Geactiveerde T-cellen kunnen of zelf kankercellen doden (cytotoxische T-cellen), of andere afweerreacties ondersteunen (helper T-cellen). Hoewel aanwezigheid van tumorspecifieke T-cellen in patiënten met eierstokkanker van invloed is op hun levensverwachting, is dit klaarblijkelijk niet voldoende om kanker te voorkomen of volledig te genezen. De verklaring hiervoor wordt gezocht in het feit dat juist die kankercellen die slecht worden herkend door het afweersysteem de beste kansen hebben om te overleven, waardoor ze uiteindelijk kunnen uitgroeien tot kwaadaardige gezwellen. Daarnaast speelt de aanwezigheid van een derde soort T-cellen wellicht een rol. Deze regulatoire T-cellen hebben als belangrijkste missie, het onderdrukken van de afweer tegen het eigen lichaam en kunnen zo dus ook tumorspecifieke T-cellen onderdrukken.




**Figuur 1** Schematische uitleg van kankerspecifieke afweerreactie. Afvalstoffen van (stervende) kankercellen (a) worden herkend en opgenomen (1) door antigeenpresenterende cellen (b). Antigeenpresenterende cellen verplaatsen zich naar de lymfeklier (2) waar antigeenspecifieke helper T-cellen (c) en cytotoxische T-cellen (d) worden geactiveerd. Geactiveerde helper T-cellen helpen o.a. bij de activatie van cytotoxische T-cellen. Cytotoxische T-cellen (d) circuleren vervolgens door het lichaam en doden cellen die het juiste antigeen presenteren (3).

## DIT PROEFSCHRIFT

Het doel van het onderzoek beschreven in dit proefschrift is tweeledig: 1) het verkrijgen van meer inzicht in de interactie tussen eierstokkanker en het afweersysteem, en 2) het uittesten van een specifieke vorm van immunotherapie in patiënten met eierstokkanker.

**Interactie tussen afweersysteem en eierstokkanker**

Zoals in het bovenstaande verklaard, bestaan er diverse soorten T-cellen. Uit **hoofdstuk 2** blijkt dat het aantal in de tumor aanwezige T-cellen sterk verschilt tussen patiënten. De aanwezigheid in tumoren van cytotoxische T-cellen, cellen die zelfstandig kankercellen kunnen doden, blijkt sterk samen te hangen met een betere levensverwachting. Ook de verhouding tussen cytotoxische T-cellen en regulatoire T-cellen (onderdrukkers van afweer tegen het eigen lichaam), blijkt een goede voorspeller te zijn voor de verwachte levensduur van patiënten met eierstokkanker. Deze invloed van T-cellen op de levensverwachting wordt gezien als een belangrijke steun in de rug voor de ontwikkeling van immunotherapie voor eierstokkanker.

In **hoofdstuk 5** wordt onderzoek beschreven dat tot doel had te achterhalen waarom in sommige tumoren veel en in andere juist weinig T-cellen aanwezig zijn. Hiervoor werd gekeken welke genen (natuurlijke eenheden van erfelijke informatie) in tumorcellen een verband lieten zien met de aan- of afwezigheid van T-cellen. Omdat genen vaak niet losstaand opereren, maar in grote verbanden, zogenaamde biologische netwerken, werd ook gekeken naar verschillen in activering van deze netwerken. Een biologisch netwerk dat vooral bij tumoren met veel T-cellen actief blijkt te zijn en dat verantwoordelijk is voor de verwerking en presentatie van antigenen, de herkenningsvlaggetjes voor afweercellen, wordt besproken in **hoofdstuk 4**. Dit hoofdstuk beschrijft hoe in een groep van 232 patiënten gekeken is naar de aanwezigheid van diverse componenten van dit netwerk in eierstokkankercellen. Antigenen zijn over het algemeen te groot om direct op het celoppervlak als vlaggetjes gehesen te worden. Daarom worden ze in de cel eerst in kleine stukjes geknipt door een eiwitcomplex. Er bestaan grofweg twee varianten van dit eiwitcomplex, het normale proteasoom en het immunoproteasoom. Het immunoproteasoom kan antigenen zo afknippen dat ze beter gehesen kunnen worden dan wanneer antigenen door het normale proteasoom worden verknipt. Nadat een antigeen in stukjes is geknipt, wordt het met behulp van vele andere eiwitten in de cel vastgekoppeld aan de vlaggenmast, een MHC klasse I molecuul. De vlag en vlaggenmast worden vervolgens samen naar het celoppervlak getransporteerd waar het geheel herkend kan worden door een afweercel. Uit het onderzoek in dit hoofdstuk blijkt dat patiënten met kankercellen die het normale proteasoom hebben en/of afwezigheid MHC klasse I op het celoppervlak een

slechtere levensverwachting hebben. Juist deze kankercellen zullen slecht herkend worden door het afweersysteem en dus ongeremd door kunnen groeien.
**Hoofdstuk 3** laat zien hoe in een groep van 329 eierstokkanker patiënten onderzoek werd gedaan naar de aanwezigheid van MHC klasse I (de vlaggenmast) op het celoppervlak, maar nu in combinatie met de aanwezigheid van een specifiek antigeen in de kankercellen, het eiwit p53. Uit dit hoofdstuk blijkt dat de levensverwachting van patiënten met veel p53-eiwit in hun cellen in combinatie met normaal MHC klasse I op het oppervlak elf maanden langer is dan die van patiënten met veel van dit antigeen, maar zonder vlaggenmasten op het celoppervlak.
Uit bovenstaande hoofdstukken blijkt dat de aanwezigheid van afweercellen in eierstokkanker belangrijk is voor de levensverwachting, maar ook dat kankercellen eigenschappen ontwikkelen om herkenning door het afweersysteem te omzeilen.
Het afweersysteem kan echter wel worden geholpen om kankercellen beter te herkennen en te vernietigen.

**Immunotherapie voor eierstokkanker**

Met immunotherapie wordt geprobeerd het natuurlijke afweerweersysteem van het lichaam zodanig te versterken en te manipuleren dat kankercellen beter worden herkend en vernietigd. In **hoofdstuk 6** wordt een overzicht gegeven van de resultaten van het onderzoek naar verschillende vormen van antigeenspecifieke immunotherapie verricht in de laatste decennia. Hoewel alle onderzochte vormen van immunotherapie in meer of mindere mate in staat blijken antigeenspecifieke afweer op te wekken, laat geen enkele van de tot dusverre uitgeteste immunotherapeutische behandelingen een remmende invloed zien op het natuurlijke beloop van eierstokkanker.
**Hoofdstuk 7** beschrijft een immunotherapeutische studie, waarbij voor het eerst 20 patiënten met teruggekomen eierstokkanker werden behandeld met een vaccin gericht tegen p53. Dit p53-SLP® vaccin bestaat uit relatief lange kunstmatig samengestelde stukken van het p53-eiwit. Deze stukken zijn zo lang dat ze niet direct door T-cellen herkend kunnen worden, maar eerst door antigeenpresenterende cellen moeten worden opgenomen (figuur 1). Het vaccin bleek veilig. In tegenstelling tot eerder geteste p53-specifieke vaccins, werden bij alle vrouwen die de vier geplande injecties kregen p53-specifieke T-cellen opgewekt. Deze studie kon geen effect op het ziekteverloop op kort termijn aantonen. In een medicijnonderzoek waarbij longkankerpatiënten werden behandeld met een p53-specifiek vaccin werd aangetoond dat patiënten die een goede p53-specifieke afweer ontwikkelden, beter reageerden op chemotherapie dan patiënten die geen p53-specifieke afweer aanmaakten. Daarom werden de patiënten uit de studie in hoofdstuk 7 nog enige tijd gevolgd. Uit **hoofdstuk 8** blijkt dat er bij eierstokkankerpatiënten behandeld met het p53-SLP® vaccin geen betere reacties op chemotherapie voor hernieuwde ziekte

werden waargenomen. Ook blijkt dat het p53-SLP® vaccin geen effect had op de levensverwachting.

## SAMENVATTING

Omdat de levensverwachting van eierstokkankerpatiënten ondanks veranderingen in de behandeling van eierstokkanker in de laatste decennia nauwelijks is verbeterd, wordt er gezocht naar nieuwe behandelvormen. Immunotherapie is een van deze nieuwe behandelstrategieën. Uit dit proefschrift blijkt dat de effecten van immunotherapie, inclusief het door ons geteste p53-SLP® vaccin, op het natuurlijke ziektebeloop vooralsnog gering zijn, ook al wordt de tumorgerichte afweer duidelijk versterkt. Dat de opgewekte afweercellen niet leiden tot vernietiging van de kankercellen, heeft ondermeer te maken met het feit dat kankercellen mechanismen ontwikkelen om herkenning en doding door het afweersysteem tegen te gaan. Mogelijkerwijs zal de combinatie van immunotherapie met middelen die het afweersysteem versterken en/of de kankercellen negatief beïnvloeden een gunstig effect op het ziektebeloop kunnen sorteren.

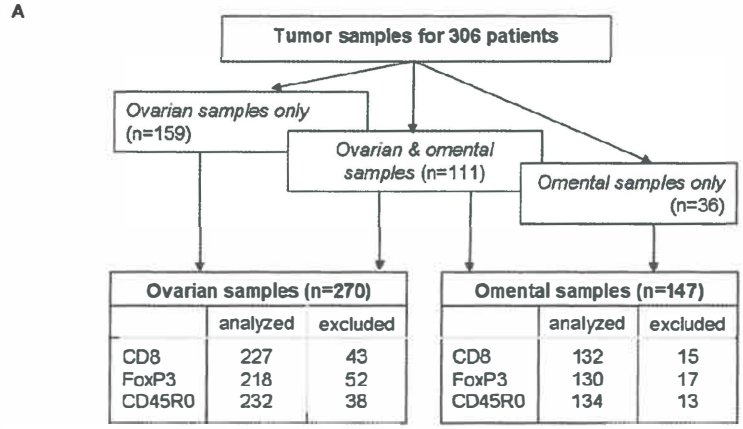
# Chapter 11

## Appendices

| | |
|---|---|
| aa | amino acid |
| Aab | auto-antibodies |
| Ab2 | anti-idiotype antibody |
| Ab3 | anti-anti-idiotype antibody |
| ANA | anti-nuclear antibodies |
| APC | antigen presenting cell |
| APPP | antigen processing and presentation pathway |
| BSA | bovine serum albumin |
| C | carboplatin |
| cCR | complete clinical remission |
| CENTRAL | Cochrane Central Register of Controlled Trials |
| CI | confidence interval |
| CNS | central nervous system |
| CR | complete response |
| CT | computerized tomography |
| CTC | common terminology criteria |
| CTL | cytotoxic T-lymphocyte |
| D | docetaxel |
| DAC | 5-aza-2'-deoxycytidine |
| DC | dendritic cell |
| DFS | disease free survival |
| DMSO | dimethyl sulfoxide |
| DSS | disease specific survival |
| ED | evidence of disease |
| EDTA | ethylenediaminetetraacetic acid |
| ER | endoplasmic reticulum |
| ERAP1 | endoplasmic reticulum aminopeptidase 1 |
| ETbR | endothelin B receptor |
| FIGO | International Federation of Gynaecology and Obstetrics |
| GCIG | Gynecologic Cancer InterGroup |
| GCP | GCP – good clinical practice |
| GMP | good manufacturing practice |
| GSEA | gene set enrichment analysis |
| H&E | haematoxylin & eosin |
| HAMA | human anti-mouse antibody |
| HLA | human leukocyte antigen |
| HNSCC | head and neck squamous cell carcinoma |
| HPV | human papilloma virus |
| HR | hazard ratio |
| IFN-γ | interferon-gamma |
| IL | Interleukin |
| IQR | interquartile range |
| iv | intravenous |
| lfu | loss to follow-up |
| LP | liposomal doxorubicin |
| LST | lymphocyte stimulation test |
| LUMC | Leiden university medical center |

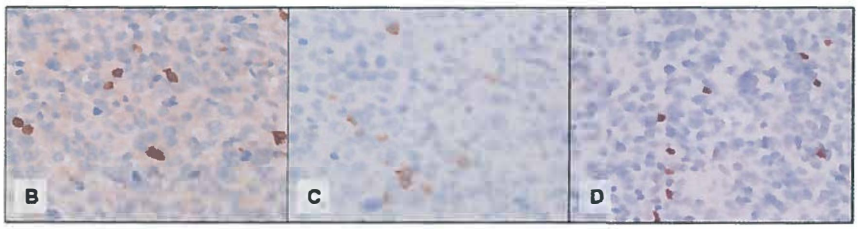
| | |
|---|---|
| MH | MH - Mantel-Haenzel |
| MHC | major histocompatibility complex |
| MRM | memory recall mix |
| na | not applicable |
| nd | not done |
| NED | no evidence of disease |
| NK-cell | natural killer cell |
| nr | not reported |
| OS | overall survival |
| P | paclitaxel |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PD | progressive disease |
| PFI | progression free interval |
| PFS | progression free survival |
| PR | partial response |
| RCT | randomised controlled trial |
| RECIST | response evaluation criteria in solid tumors |
| SD | standard deviation |
| SD | stable disease |
| SEM | standard error of mean |
| SI | stimulation index |
| SLP | synthetic long peptide |
| sc | subcutaneous |
| TAP | transporter associated with antigen processing |
| TCGF | T-cell growth factor |
| TGF | transforming growth factor |
| Th | T-helper |
| TIL | tumour-infiltrating lymphocyte |
| TLR | Toll-like receptor |
| TMA | tissue microarray |
| TNF | tumour necrosis factor |
| TTP | time to progression |
| TTR | time to relapse |
| UMCG | UMCG - university medical center Groningen |
| UNK | unknown |
| WHO | World Health Organization |

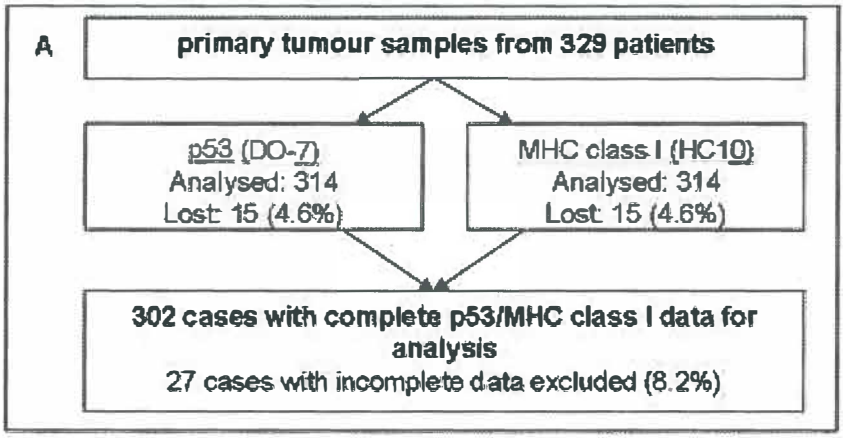
A

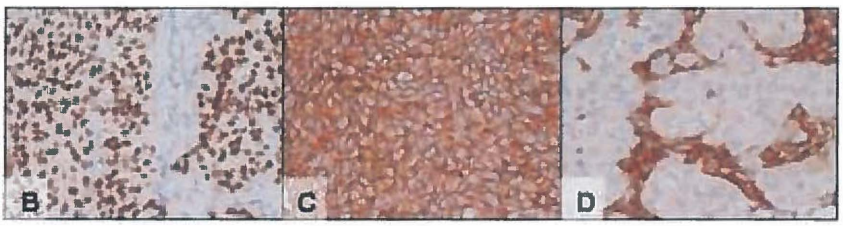


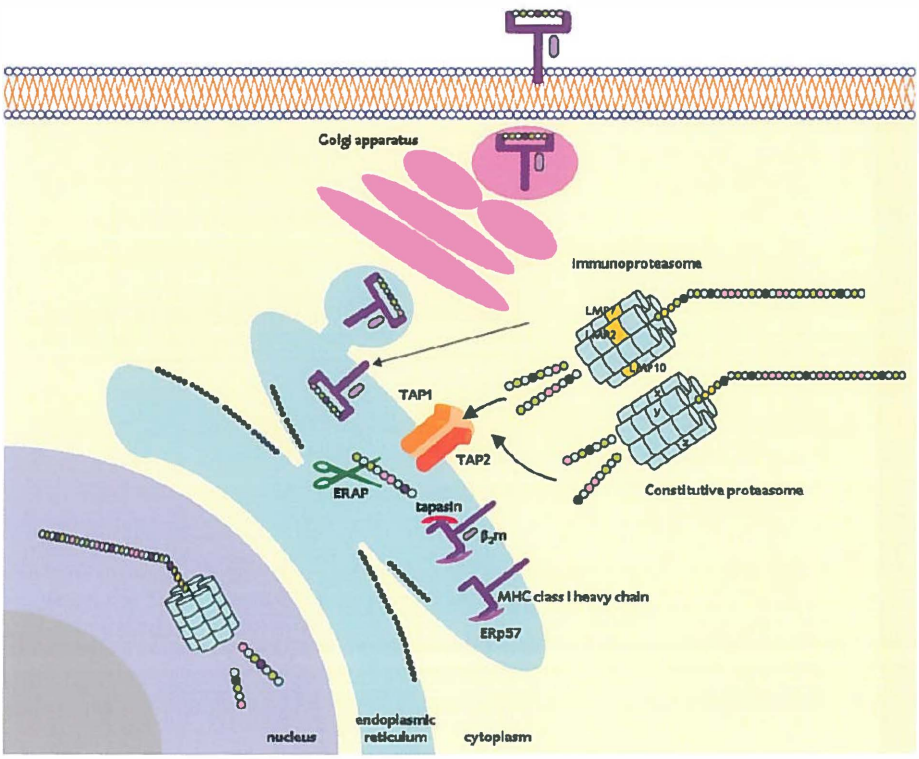

| Ovarian samples (n=270) | | |
|---|---|---|
| | analyzed | excluded |
| CD8 | 227 | 43 |
| FoxP3 | 218 | 52 |
| CD45R0 | 232 | 38 |

| Omental samples (n=147) | | |
|---|---|---|
| | analyzed | excluded |
| CD8 | 132 | 15 |
| FoxP3 | 130 | 17 |
| CD45R0 | 134 | 13 |

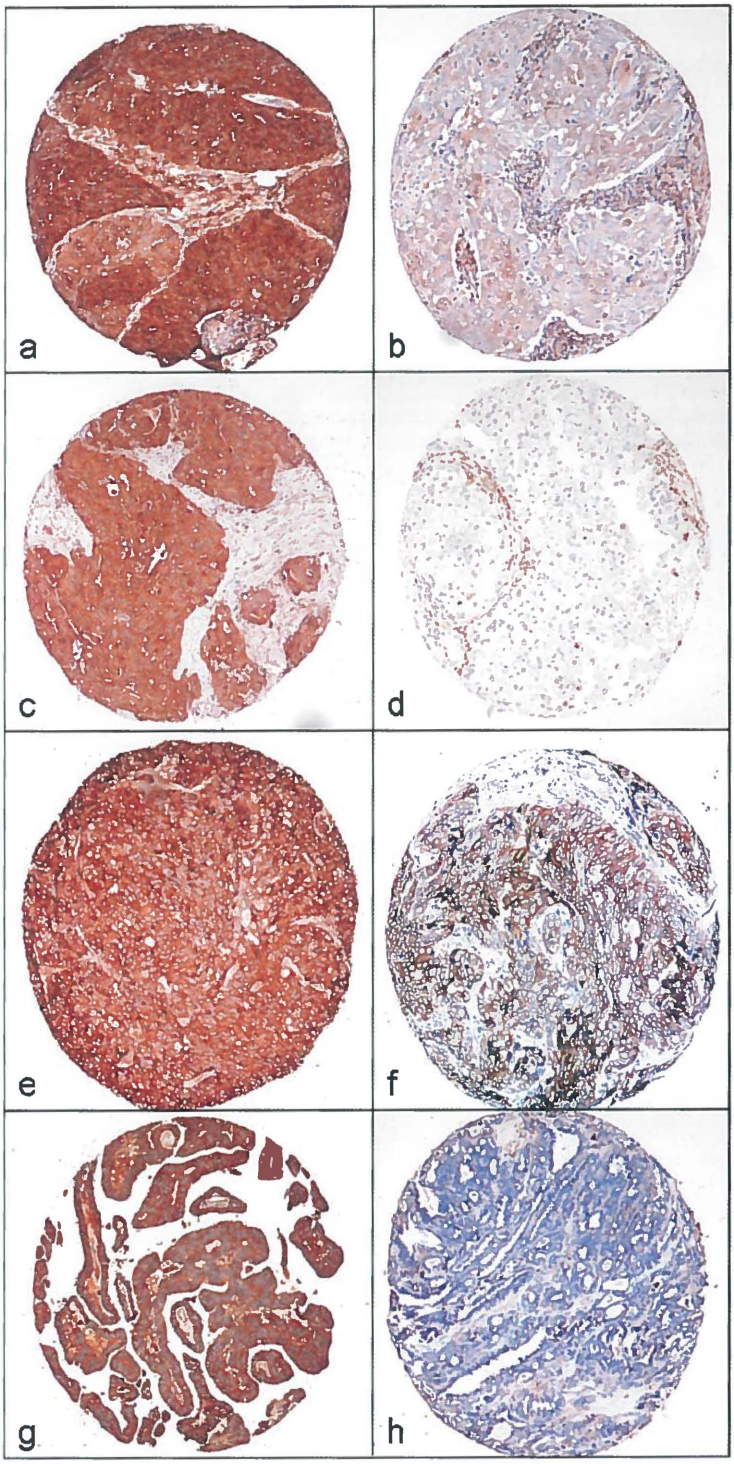





Chapter 4, Fig.1


a
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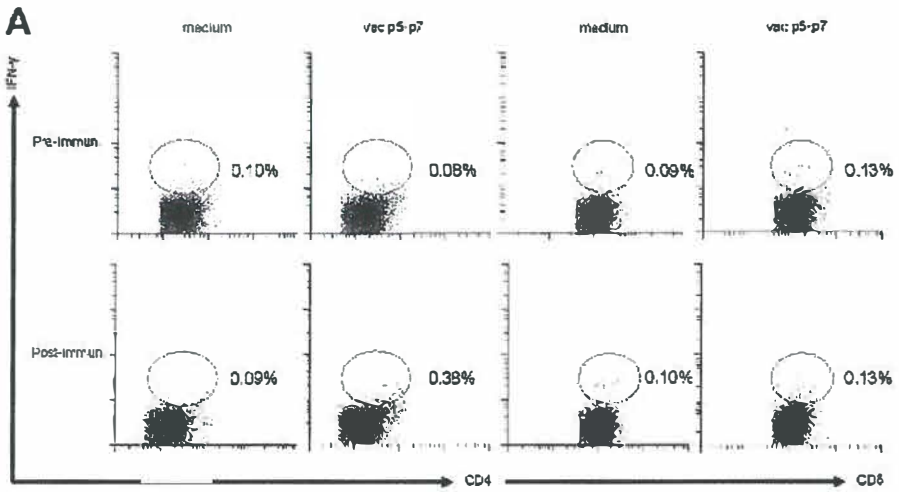
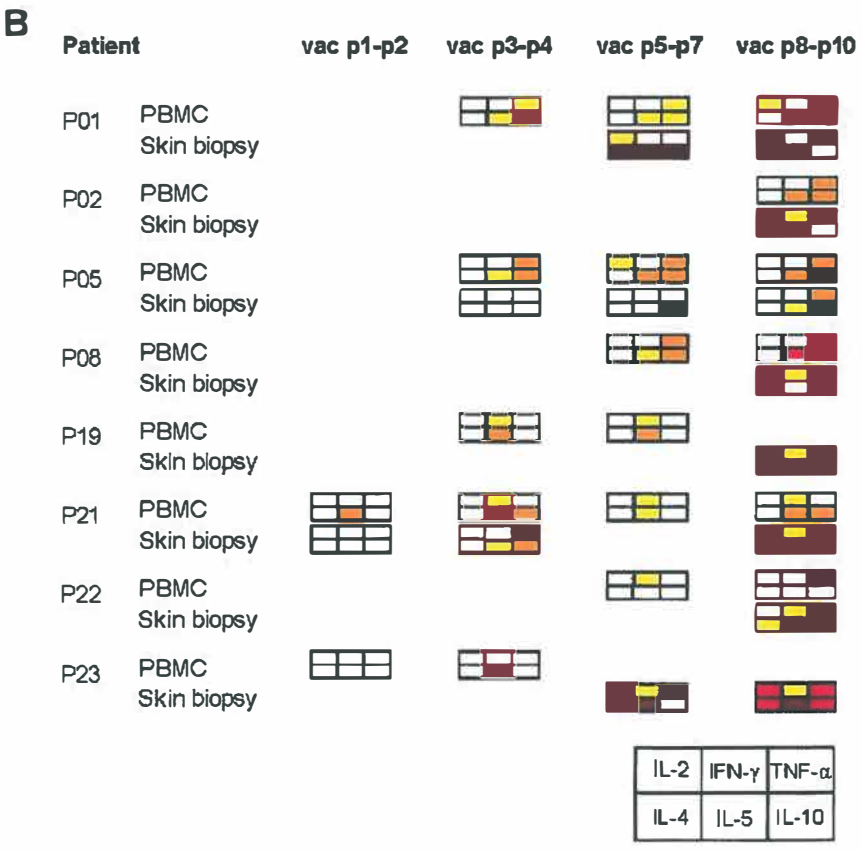
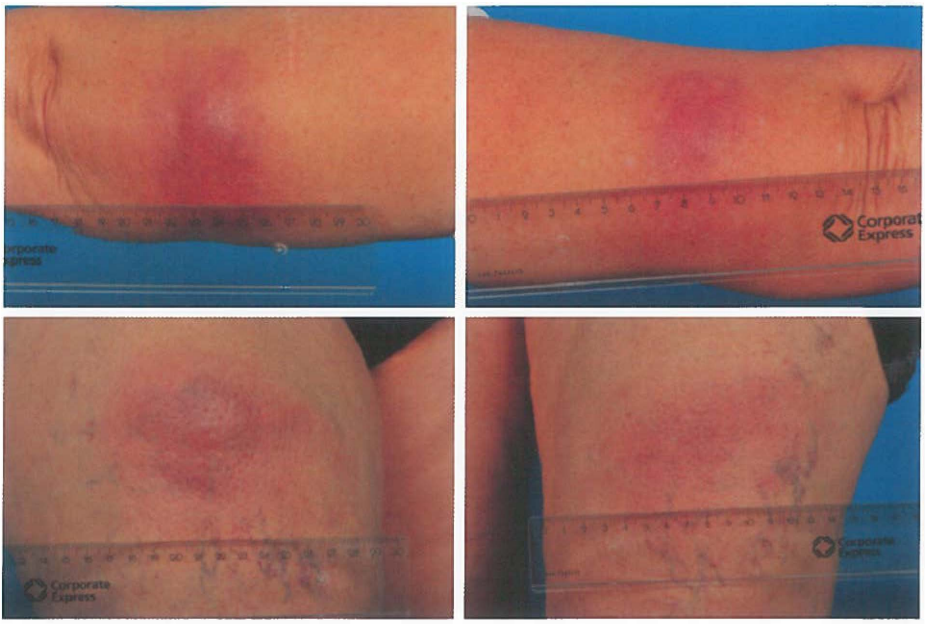
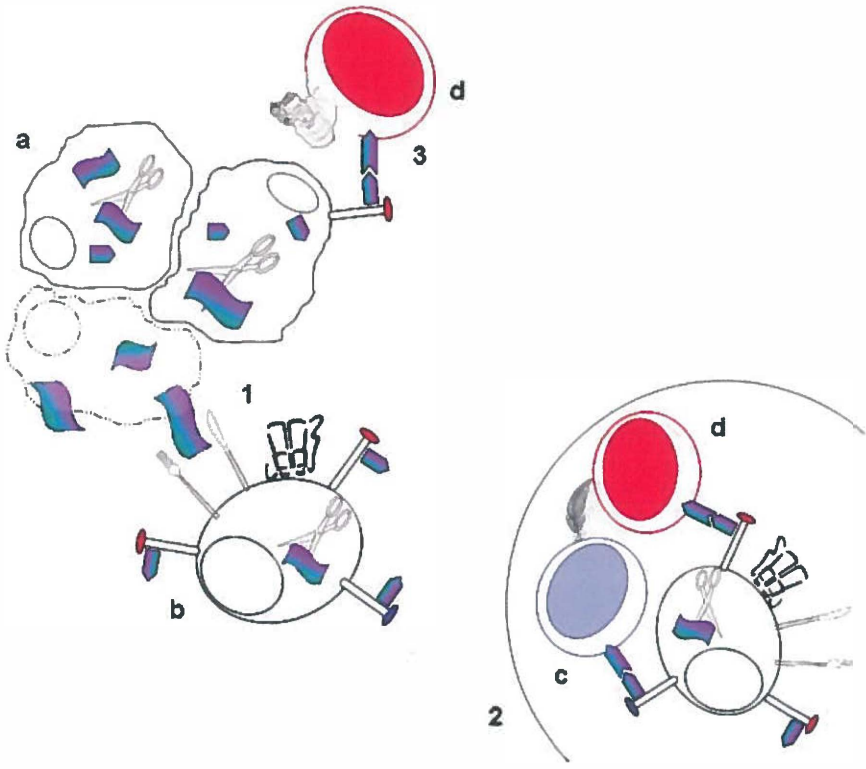


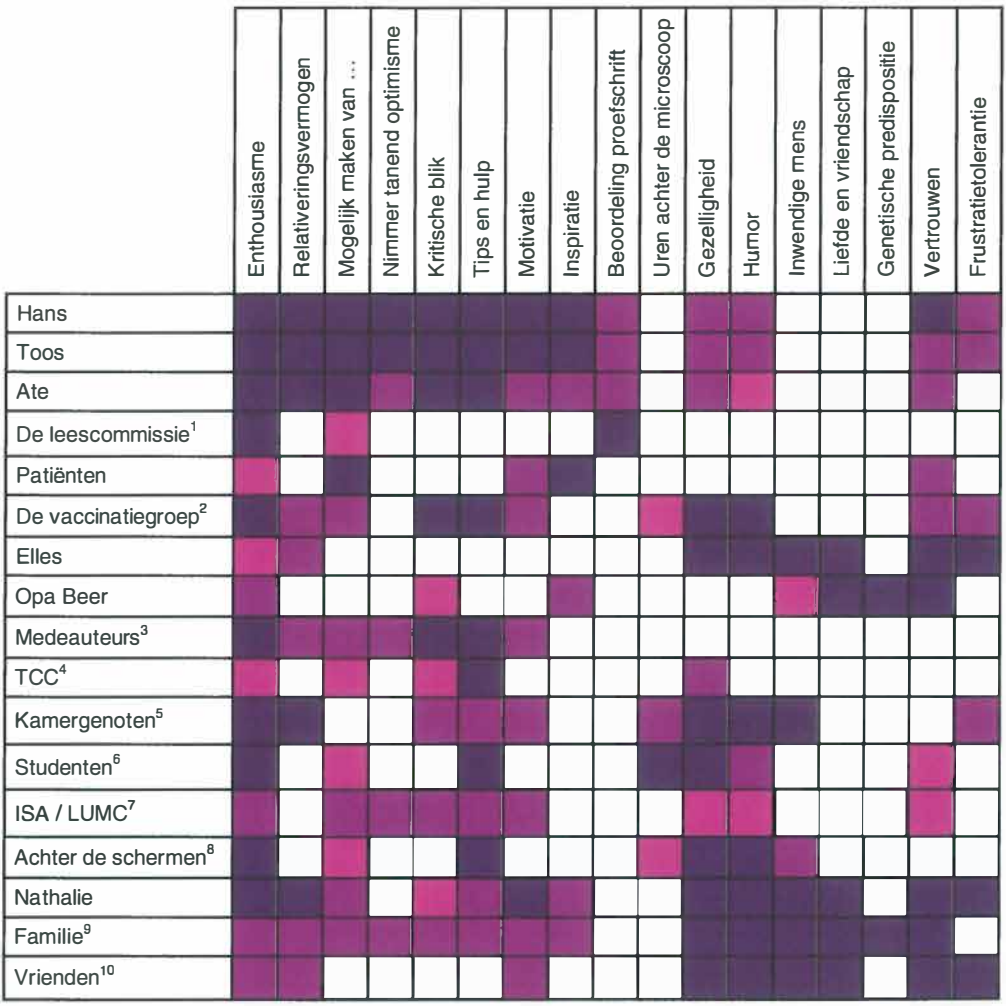

Chapter 7, Fig.2



Chapter 8, Fig.1




Chapter 10, Fig.1




**Figuur Dankwoord** Heatmap waarbij kleurintensiteit mate van bijdrage aan dit proefschrift weergeeft*. ■ fantastisch ; ■ geweldig; ■ goed; □ wellicht volledig ten onrechte niet ingevuld#

[1] Gemma Kenter, Stephen Schoenberger, Pax Willemse
[2] Baukje-Nynke, Annechien, Ineke, Ute, Renee, Henriette
[3] In het bijzonder: Marike Boezen, Harry Hollema, Rudolf Fehrmann, Rinze Wolf
[4] In het bijzonder: Ilse Snieders, Janneke Bergsma, Nic Veeger, Myke Mol, Denise Mailly
[5] Pauline, Melanie, Esther, Justine, Monique, Maaike, Jasper, Claudia, Renee, Renske
[6] Mariska, Sebastian, Anna, Astrid, Serge, Marloes, Anneline, Yvette
[7] In het bijzonder: Sjoerd v.d. Burg, Kees Melief, Sanne Weijzen, Mark Krul, Johan Frieling
[8] In het bijzonder: Bert, Gerard, Klaske, Harry, poli O&G, secretaresses O&G
[9] Papa, mama, Janneke & Sebastian, Diederik & Babette, Opa en Oma Leffers, Opa en Oma Dekker
[10] In het bijzonder: Fen, Cynthia, Carla, Karin, Kelly, Margreet, Jerry, Ellen, Brigit, Tineke, Inge, Astrid, Nynke, Melissa en Sigrid

** adapted with permission from Dreezens E. The missing link: the relationship between values and attitudes. Maastricht 2009*
#NB. Experiment is niet in triplo uitgevoerd. Interne en externe validiteit staan derhalve ter discussie.

Lambeck AJ, Crijns AP, Leffers N, Sluiter WJ, ten Hoor KA, Braid M, van der Zee AG, Daemen T, Nijman HW, Kast WM. Serum cytokine profiling as a diagnostic and prognostic tool in ovarian cancer: a potential role for interleukin 7. Clin Cancer Res 2007; 13:2385-2391.

Lambeck A, Leffers N, Hoogeboom BN, Sluiter W, Hamming I, Klip H, ten Hoor K, Esajas M, van Oven M, Drijfhout JW, Platteel I, Offringa R, Hollema H, Melief K, van der Brug S, van der Zee A, Daemen T, Nijman H. P53-specific T cell responses in patients with malignant and benign ovarian tumors: Implications for p53 based immunotherapy. Int J Cancer 2007; 121:606-614.

Leffers N, Lambeck AJA, de Graeff P, Bijlsma AY, Daemen T, van der Zee AG, Nijman HW. Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. Gynecol Oncol 2008; 110:365-373.

Leffers N, Helfrich W, Boezen HM, Cohlen BJ, Melief CJ, Nijman HW. Antigen-specific active immunotherapy for ovarian cancer (protocol). Cochrane Database of Systematic Reviews 2008, issue 3.

Leffers N, Gooden MJ, de Jong RA, Hoogeboom BN, ten Hoor KA, Hollema H, Boezen HM, van der Zee AG, Daemen T, Nijman HW. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. Cancer Immunol Immunother 2009; 58:449-459.

Leffers N, Gooden MJ, Mokhova AA, Kast WM, Boezen HM, ten Hoor KA, Hollema H, Daemen T, van der Zee AG, Nijman HW. Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. Gynecol Oncol 2009; 113:256-263.

De Jong RA, Leffers N, Boezen HM, ten Hoor KA, van der Zee AG, Hollema H, Nijman HW. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. Gynecol Oncol 2009;114:105-10.

Leffers N, Lambeck AJ, Gooden MJ, Hoogeboom BN, Wolf R, Hamming LE, Hepkema BH, Willemse PH, Molmans BH, Hollema H, Drijfhout JW, Sluiter WJ, Valentijn AR, Fathers LM, Oostendorp J, van der Zee AG, Melief CJ, van der Burg SH, Damen T, Nijman HW. Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial. Int J Cancer 2009;125:21-4-2113.

Leffers N, Daemen T, van der Zee AGJ, Nijman HW. Multimodality treatment warranted for ovarian cancer: immunotherapy, a prerequisite to improve prognosis for this vicious disease. Immunotherapy 2009;1:163-5.

Bijen CM, Bantema-Joppe EJ, de Jong RA, Leffers N, Mourits MJE, Eggink HF, van der Zee AGJ, Hollema H, de Bock GH, Nijman HW. The prognostic role of classical and non-classical MHC class I expression in endometrial cancer. Int J Cancer 2009 Sep 2.

Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief CJ, Nijman HW. Antigen-specific active immunotherapy for ovarian cancer (review). Submitted to Cochrane Database of Systematic Reviews.

Ninke Leffers werd geboren te Amsterdam op 14 mei 1978. Het grootste deel van haar jeugd bracht zij door in Maastricht, waar zij in 1996 het VWO diploma behaalde. Na haar middelbare schooltijd, bracht zij in 1996-1997 negen maanden door bij Drs. Tarek Meguid in het Onadjokwe Lutheran Hospital te Ondangwa, Namibië, waar haar overtuiging arts te willen worden alleen maar toenam. Omdat zij werd uitgeloot voor de studie geneeskunde, studeerde zij van 1997-1999 psychologie aan de Universiteit Maastricht. In een poging de biologie niet uit het oog te verliezen, startte zij in 1998 tevens met het propedeusejaar van de studie Gezondheidswetenschappen aan de Universiteit Maastricht. Na dat studiejaar werd zij alsnog geplaatst voor de studie geneeskunde. Zij verhuisde naar Groningen, alwaar zij cum laude het doctoraal en artsexamen aflegde. Nadien heeft zij enkele maanden als AGNIO gewerkt op de afdeling Gynaecologie en Obstetrie van het Martini Ziekenhuis te Groningen. Begin 2006 startte zij met het promotieonderzoek dat leidde tot dit proefschrift.

Ninke start op 1 december 2009 met de opleiding tot gynaecoloog in het cluster Groningen. Ze woont samen met Nathalie, met wie ze haar passie voor viervoeters, koken, klussen, reizen en natuur deelt.